This is a pre- or post-print of an article published in

Franzen, L., Vidlářová, L., Kostka, K.-H., Schaefer, U.F., Windbergs, M.

Freeze-drying as a preserving preparation technique for in vitro testing of human skin

(2013) Experimental Dermatology, 22 (1), pp. 54-56.

Freeze drying as a preserving preparation technique for in vitro testing of human skin

[1]Lutz Franzen, [1,2]Lucie Vidlářová, [3]Karl-Heinz Kostka, [1]Ulrich F. Schaefer, [1,4,5]Maike Windbergs

[1]*Department of Biopharmaceutics and Pharmaceutical Technology, Saarland University, Saarbruecken, Germany*

[2]*Department of Pharmaceutical Technology, Faculty of Pharmacy, Hradec Králové, Charles University, Prague, Czech Republic*

[3]*Department of Plastic and Hand Surgery, Caritas-Krankenhaus, Lebach, Germany*

[4]*PharmBioTec GmbH, Saarbruecken, Germany*

[5]*Helmholtz-Institute for Pharmaceutical Research Saarland, Saarbruecken, Germany*

Corresponding author:

Dr. Maike Windbergs

Department for Biopharmaceutics and Pharmaceutical Technology

Saarland University, Campus A4.1

66123 Saarbruecken

m.windbergs@mx.uni-saarland.de

Phone: +49 681 302 2358

Fax: +49 681 302 4677

ABSTRACT

In vitro testing of drugs with excised human skin is a valuable prerequisite for clinical studies. However, the analysis of excised human skin presents several obstacles. Ongoing drug diffusion, microbial growth and changes in hydration state influence the results of drug penetration studies. In this work, we evaluate freeze drying as a preserving preparation method for skin samples to overcome these obstacles. We analyze excised human skin before and after freeze drying and compare these results with human skin in vivo. Based on comprehensive thermal and spectroscopic analysis, we demonstrate comparability to in vivo conditions and exclude significant changes within the skin samples due to freeze drying. Furthermore, we show that freeze drying after skin incubation with drugs prevents growth of drug crystals on the skin surface due to drying effects.

In conclusion, we introduce freeze drying as a preserving preparation technique for in vitro testing of human skin.

BACKGROUND

For rational development of novel drug delivery strategies via the skin, in vitro testing of drug behaviour and penetration into excised human skin is a mandatory prerequisite for clinical studies. As the accessibility of drug penetration data within the different skin layers, apart from plasma concentration, is severely limited in human in vivo studies, detailed and spatially resolved information about the extent of drug penetration and kinetics can solely be gained by in vitro testing.

Even though the in vitro methodology is well established, the handling of excised human skin for in vitro testing reveals many obstacles (1-3). Drug penetration studies generally follow strict time intervals for sampling. However, penetration within the tissue continues after termination of drug incubation and thus time consuming analysis can lead to misinterpretations of drug penetration kinetics (4). In addition, microbial growth facilitated by long term experiments and changes in the hydration state of the sample influence the results. Based on this situation, there is a high demand for a technique which preserves the skin samples.

QUESTIONS ADDRESSED

In this study, we evaluate freeze drying as a preserving preparation technique for in vitro testing of human skin. To assure applicability of freeze dried skin samples for in vitro analysis, the absence of structural changes within the skin tissue has to be verified in a comparison with human skin in vivo. Furthermore, detectability and the behaviour of drugs within the tissue have to be investigated.

EXPERIMENTAL DESIGN

Human skin preparation

Human skin was obtained from plastic surgery of female Caucasians (Department of Plastic and Hand Surgery, Caritaskrankenhaus, Lebach, Germany). Only abdominal skin was used. After excision the stratum corneum was cleaned with purified water and the fatty tissue was removed with a scalpel. The skin was stored in impermeable polyethylene bags at -26 °C. First, punches of 25 mm in diameter were taken from the frozen skin and thawed to room temperature on a filter paper soaked with phosphate buffered saline (composed of 0.2 g potassium chloride, 8.0 g sodium chloride, 1.44 g disodium hydrogen phosphate dehydrate and 0.2 g potassium dihydrogen phosphatein 1 l of purified water). To separate the full-thickness skin in dermis and epidermis, the thawed sheets were immersed in 60 °C warm purified water for 90 s. Subsequently, the epidermis was peeled of using forceps. The dermis was disposed and the heat separated epidermis was dry between two filter papers using a pressure roll.

To separate stratum corneum and epidermis, the sheets were incubated in 0.15 % (m/m) solution of trypsin in phosphate-buffered saline at 37 °C. After 24 h the remnants of viable epidermis were removed in purified water and the remaining stratum corneum sheets were blotted dry.

To exclude interindividual variability fresh and freeze dried samples were taken from the same donor. A total of three donors were analysed.

In vivo data were recorded from the palm of hand using the instruments and settings listed below. All tested individuals gave written consent to participate in this study. The study was approved by the Ethical Commission of the Department of Plastic and Hand Surgery, Caritaskrankenhaus, Lebach, Germany (204/08).

Skin incubation experiments

For incubation experiments heat separated epidermis and stratum corneum were incubated in a petri dish filled with caffeine solution (12.5 mg/ml) in phosphate-buffer for 16 h. After incubation the skin was patted dry and analysed or frozen for freeze drying immediately.

Freeze drying procedure

For freeze drying stratum corneum and heat separated epidermis were placed on flat Teflon sheets and frozen immediately after preparation to -80 °C. Freeze drying was performed in a shelf freeze dryer with a condenser temperature of -82 °C (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). Primary drying was performed for at least 48 h with a controlled shelf temperature of 16 °C and 0.2 mbar pressure. To assure complete sublimation a secondary drying step at 26 °C shelf temperature and 0.08 mbar was conducted for 1 h. After freeze drying the skin samples were stored in a desiccator over silica gel. The complete removal of water is indicated in a total loss of flexibility of the skin sheets and a stiff and fragile consistency.

Transmittance-polarised-light microscopy

Pictures of the skin samples were taken by a transmittance-polarised-light microscope (BH-2, Olympus, Hamburg, Germany). A Phase-difference-plug (530nm) transformed the grade of interference into a colour profile. Pictures were captured at 4 times magnification (DPlan 4, N.A. 0.1, Olympus, Hamburg, Germany) using an digital microscope camera (Moticam 2300, Motic Deutschland GmbH, Wetzlar, Germany).

Differential scanning calorimetry (DSC)

For DSC measurements samples were sealed in hermetic aluminium pans (Hermetic Pans, TA Instruments, USA). After equilibration for 2 min at 25 °C, temperature ramps in the range of 25-120 °C were performed with a heating rate of 10 °C/min (DSC Q100, TA Instruments, USA). Three measurements from each donor and each treatment were averaged.

Confocal Raman microscopy

Ramanspectra were recorded using a confocal Raman microscope (alpha300R+, WITecGmbH, Ulm, Germany). The excitation source was a diode laser with a wavelength of 785 nm adjusted to a power of 25 mW on the sample surface. Singlespectra were recorded using a 50x objective (Epiplan Neofluar, Zeiss, Germany) with a numeric aperture of 0.8. A confocal pinhole of 100 µm rejected signals from out-of-focus regions. Raman spectra were recorded in the range of 400-1780 cm-1 with a spectral resolution of 4 cm-1 and 10 seconds acquisition time with 10 accumulations. Mean spectra from three different measurement spots were derived. The data were processed using WITec Project Plus software (WITec GmbH, Ulm,Germany). After cosmic ray removal the spectral baseline was corrected using a polynomic fit. All spectra were normalised to the most intense peak (1430-1480 cm-1 representing v(C-H)).

Infrared-Spectroscopy (IR)

Infrared spectroscopy analysis was performed with an attenuated total reflectance (ATR) unit (Spectrometer 400 ATR-IR, Perkin Elmer, USA). Spectra were recorded in the range

of 650-4000 cm-1 with 10 accumulations. The skin sheets were placed directly on the diamond crystal and pressure was applied.

RESULTS

To investigate thermal properties of excised skin samples and reveal potential changes in their structural organisation, stratum corneum (SC) and heat separated epidermis (HSE) were analysed by differential scanning calorimetry (DSC) before and after freeze drying. Isolated SC (Figure 1 A) shows specific endothermic peaks in accordance with the literature (5). Freeze dried samples exhibit more pronounced peaks with a slight temperature shift due to the absence of water in the tissue (6). Rehydration of the freeze dried samples in controlled humidity restores the original thermogram peaks demonstrating this water effect (data not shown). As expected, thermograms of HSE did not exhibit any thermic events in the analysed temperature range, due to the low lipid concentration and a broad protein melting peak of keratin (7, 8).

Infrared (IR) spectroscopy as a valid technique to identify structural changes exposes no significant differences between human skin in vivo, fresh and freeze dried HSE and SC, except for their water content (Figures 1 B and C). The water related peak is embodied by the *v*(OH) band around 3250 cm^{-1} (9, 10). No additional peaks or peak shifts indicating chemical changes or conformational alterations can be observed for freeze dried samples. The higher lipid content in SC compared to HSE can be visualised by higher intensities in the spectral region representing *v*(CH) between 2800 cm^{-1} and 3060 cm^{-1} (11, 12).

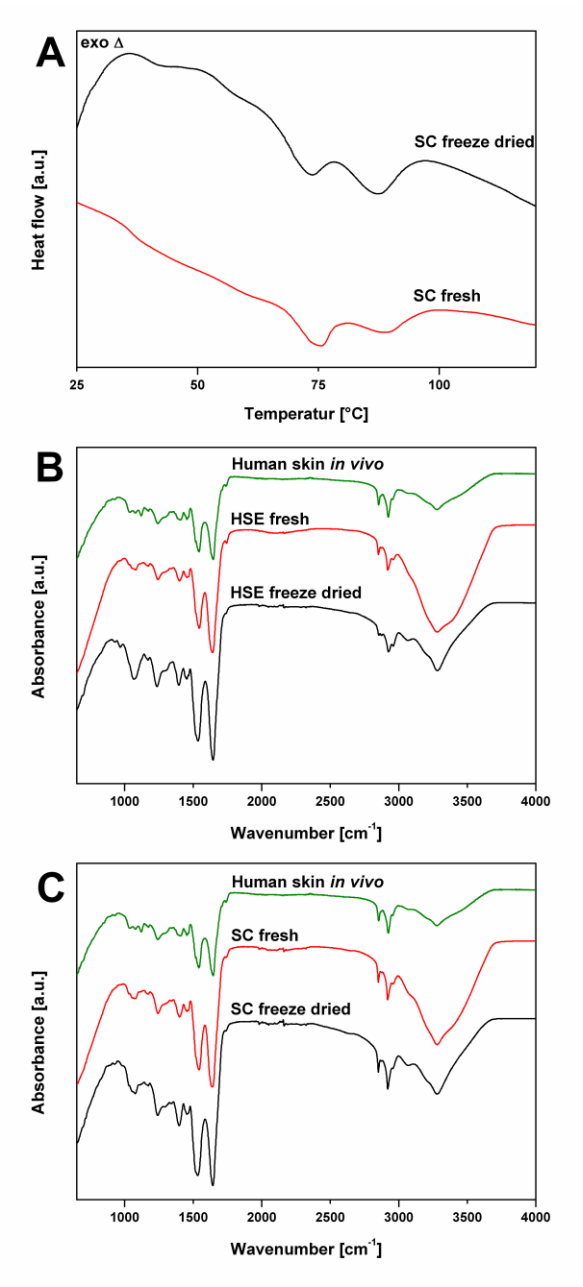

Figure 1: A DSC thermograms of stratum corneum (SC) and **B C** IR spectra of heat separated epidermis (HSE) and stratum corneum before and after freeze drying compared to human skin in vivo.

Furthermore, Raman spectroscopy analysis as an upcoming analytical technique for skin research is used to demonstrate optical similarity between human skin in vivo, fresh

and freeze dried excised skin. Spectra from fresh and freeze dried SC (Figure 2 A) and HSE samples (Figure 2 B) were compared to human skin in vivo.

To gain statistically valid information regarding the similarity of freeze dried and fresh skin samples, a spectra subtraction analysis was performed. This includes the subtraction of the intensity values of two spectra resulting in a graphical illustration of the spectral difference. The absolute area under the curve (AUC) of this plot is employed to quantify the discrepancy between the subtracted spectra.

To determine a zero value representing the intra-individual variability, three spectra recorded from the same skin sample on different positions were analysed. The result was a mean AUC of 70.7 cts*cm^{-1} with a 95 % confidence interval of up to 94.6 cts*cm^{-1}.

In the next step the spectra of freeze dried and fresh SC (Figure 2 C) and HSE (Figure 2 D), respectively were subtracted as well. The determined AUCs for SC (65.1 cts*cm^{-1}) and HSE (79.4 cts*cm^{-1}) were both covered by the 95% confidence interval of the reference. Therefore, it can be concluded that the spectral difference between freeze dried and fresh skin samples is not statistically different from the individual spectral variability.

Based on this comprehensive analysis using thermal as well as spectroscopic techniques, comparability to in vivo conditions can be proved and significant changes within the skin samples due to freeze drying are excluded.

As a further issue, the uncontrolled crystallisation of drugs in skin samples due to supersaturation during drying is addressed. Human skin samples, incubated in caffeine solution were analysed by polarised light microscopy and confocal Raman microscopy directly after incubation and after freeze drying. Already hours after incubation caffeine crystals appear on the skin. Figure 2 E depicts polarised-transmitted-light microscopic

pictures of skin samples freeze dried and non-freeze dried (I and II HSE, III and IV SC). Figure 2 E I and III expose the presence of crystalline caffeine-hydrate in typical needle shape in skin samples. No such crystals are observed in samples freeze dried immediately after incubation (Figure 2 E II and IV). Thus, freeze drying preserves the molecular disperse distribution and avoids large crystal growth due to slow drying effects (13).

Figure 2 F exhibits Raman spectra of crystalline caffeine as observed in incubated skin samples and caffeine in freeze dried samples. The higher intensity of caffeine bands in crystalline state outshines the skin bands. In freeze dried skin, the caffeine peak at 553 cm^{-1} (δ(O=C-N)) (14) is clearly detectable and not interfering with any skin related peaks. This facilitates identification and quantification of drug inside the skin.

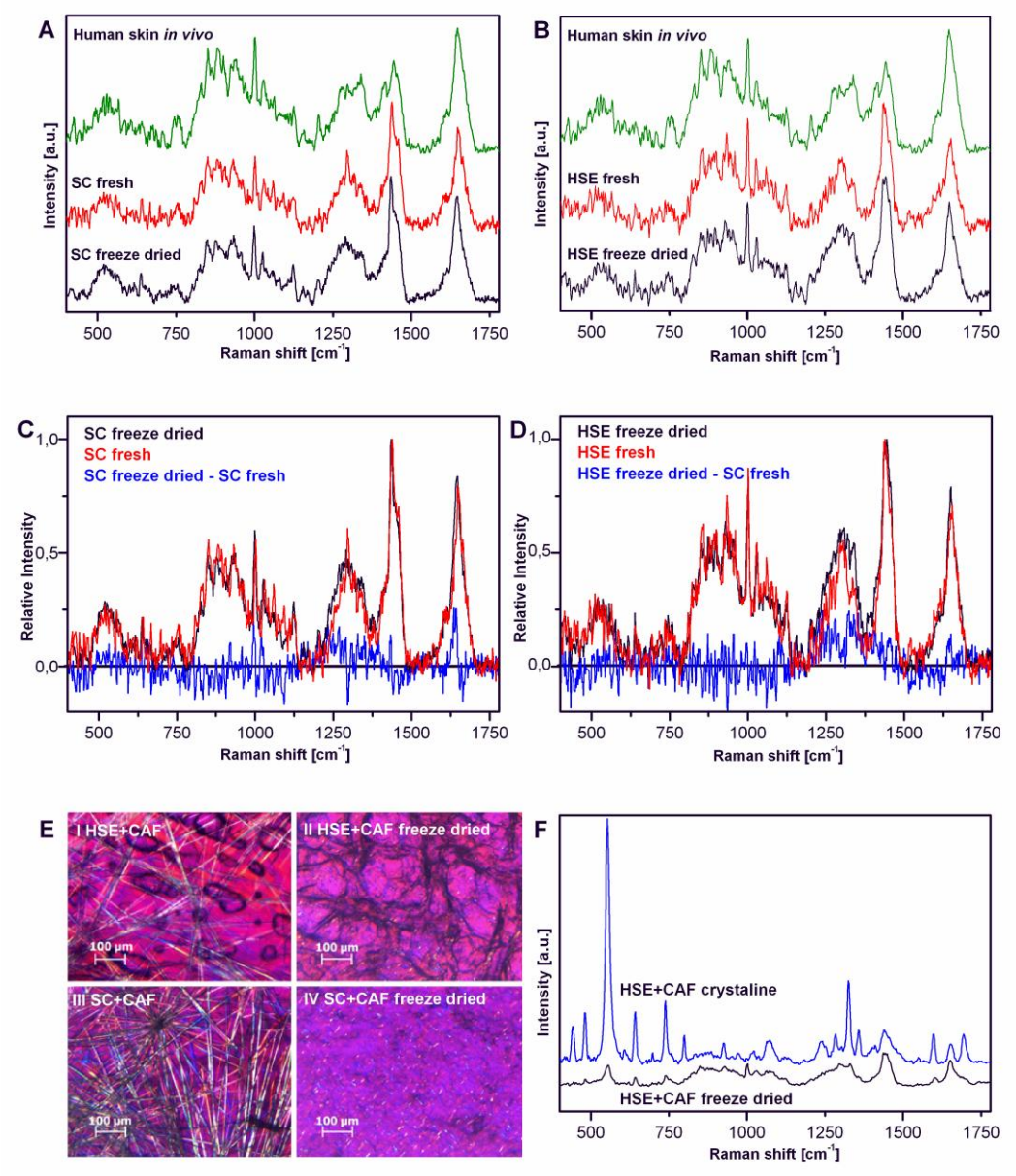

Figure 2: Raman spectra obtained from human skin in vivo compared to **A** stratum corneum (SC) and **B** heat separated epidermis (HSE) before and after freeze drying. Raman spectra overlay from fresh and freeze dried SC **C** and HSE **D** and the resulting subtraction spectrum. **E** Polarised-light microscopic pictures of heat separated epidermis (**I** and **II**) and stratum corneum (**III** and **IV**) incubated with caffeine with and without freeze drying. **F** Raman spectra of crystalline caffeine in HSE and caffeine in freeze dried HSE.

CONCLUSIONS

Freeze drying has been successfully introduced as a suitable preserving preparation technique for in vitro testing of human skin. We highlight that optical similarity to human skin in vivo remains after excision and neither HSE nor SC samples are chemically affected by the freeze drying procedure. Besides easier sample handling and prevention of microbial growth, the technique instantly preserves the drug diffusion status, thus avoiding drug crystallization and misinterpretation of penetration kinetics.

ACKNOWLEDGEMENTS

L. Franzen, M. Windbergs and L. Vidlářová performed the experiments and analysed the data. M. Windbergs and U.F. Schaefer designed the research study. K.-H. Kostka provided the human skin samples. L. Franzen and M. Windbergs wrote the manuscript.

CONFLICT OF INTEREST

The authors state no conflict of interest.

REFERENCES

1. Hahn T, Selzer D, Neumann D, Kostka K H, Lehr C M, Schaefer U F. Influence of the application area on finite dose permeation in relation to drug type applied. Exp Dermatol 2012: 21: 233-235.

2. Wagner H, Kostka K H, Lehr C M, Schaefer U F. Drug distribution in human skin using two different in vitro test systems: Comparison with in vivo data. Pharm Res 2000: 17: 1475-1481.

3. Henning A, Schaefer U F, Neumann D. Potential pitfalls in skin permeation experiments: Influence of experimental factors and subsequent data evaluation. Eur J Pharm Biopharm 2009: 72: 324-331.

4. Piot O, Tfayli A, Manfait M. Confocal Raman microspectroscopy on excised human skin: uncertainties in depth profiling and mathematical correction applied to dermatological drug permeation. J Biophotonics 2008: 1: 140-153.

5. Kaushik D, Michniak-Kohn B. Percutaneous Penetration Modifiers and Formulation Effects: Thermal and Spectral Analyses. AAPS PharmSciTech 2010: 11: 1068-1083.

6. Vanduzee B F. Thermal-Analysis of Human Stratum-Corneum. J Invest Dermatol 1975: 65: 404-408.

7. Babita K, Kumar V, Rana V, Jain S, Tiwary A K. Thermotropic and spectroscopic behavior of skin: relationship with percutaneous permeation enhancement. Curr Drug Deliv 2006: 3: 95-113.

8. Al-Saidan S M, Barry B W, Williams A C. Differential scanning calorimetry of human and animal stratum corneum membranes. Int J Pharm 1998: 168: 17-22.

9. Barry B W, Edwards H G M, Williams A C. Fourier-Transform Raman and Infrared Vibrational Study of Human Skin - Assignment of Spectral Bands. J Raman Spectrosc 1992: 23: 641-645.

10. Nakagawa N, Naito S, Yakumaru M, Sakai S. Hydrating effect of potassium lactate is caused by increasing the interaction between water molecules and the serine residue of the stratum corneum protein. Exp Dermatol 2011: 20: 826-831.

11. Boncheva M, Damien F, Normand V. Molecular organization of the lipid matrix in intact Stratum corneum using ATR-FTIR spectroscopy. BBA-Biomembranes 2008: 1778: 1344-1355.

12. Berthaud F, Boncheva M. Correlation between the properties of the lipid matrix and the degrees of integrity and cohesion in healthy human Stratum corneum. Exp Dermatol 2011: 20: 255-262.

13. de Waard H, Hinrichs W L J, Frijlink H W. A novel bottom-up process to produce drug nanocrystals: Controlled crystallization during freeze-drying. J Control Release 2008: 128: 179-183.

14. Baranska M, Proniewicz L M. Raman mapping of caffeine alkaloid. Vib Spectrosc 2008: 48: 153-157.