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Research Paper

Assessment of the "Skin Reservoir" of Urea by Confocal Raman Microspectroscopy and Reverse Iontophoresis *in vivo*

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Purpose. To demonstrate the "skin reservoir" of urea by confocal Raman microspectroscopy *in vivo* and to evaluate its impact on the non-invasive monitoring of the analyte by reverse iontophoresis.
Methods. Urea was extracted iontophoretically over a 2-h period across the skin of adult volunteers and patients with chronic kidney disease. Confocal Raman microspectroscopic profiles of skin were recorded before and after 30 min of current application.
Results. Urea extraction was higher at the beginning of current passage, but then decreased to achieve stable values after 2 h of iontophoresis. After 30 min of iontophoresis, the Raman spectra highlighted a clear depletion of urea at the surface of the skin. Lactate distribution was also modified both at the surface and deeper into the skin.
Conclusions. A source of urea in the skin, unrelated to the concentration circulating in the blood, was strongly suggested by extracted urea flux observed over time and by the Raman spectroscopy. This "urea reservoir" must be removed before systemic urea levels can be non-invasively monitored by reverse iontophoresis.

KEY WORDS: confocal raman microspectroscopy; reverse iontophoresis; skin reservoir; urea.

INTRODUCTION

The potential of reverse iontophoresis to non-invasively monitor chronic disease has been demonstrated recently by the development of the GlucoWatch G2 Biographer®, which can provide a continuous measure of blood glucose (1). Reverse iontophoresis involves transdermal application of a low current (<0.5 mA/cm^2) to enhance the passage of ions and of uncharged, polar molecules across the skin (2).

Monitoring systemic urea levels is useful to diagnose chronic renal disease and to track the progress of hemodialysis in patients with kidney failure. The non-invasive use of iontophoresis for this purpose has been investigated *in vitro* and the role of an interfering "urea skin reservoir" has been reported (3). It seems that this reservoir must be depleted before a linear correlation between extracted fluxes and subdermal concentrations of urea can be established. To confirm the presence of this urea depot *in vivo*, confocal Raman microspectroscopy was used to assess changes in urea distribution in the stratum corneum (skin's outermost and least permeable layer) over the course of iontophoresis.

Raman spectroscopy is a well known optical method to assess molecular composition, structure and interactions in a sample. This non-destructive method is based on inelastic light scattering and can be directly applied with little or no sample preparation. Raman has been used previously to study human skin (4–8). A more recent development is *in vivo* confocal Raman microspectroscopy, which provides detailed information about the molecular composition of human skin *in situ* with high spatial resolution (9,10). A spatial resolution of 5 μm or better can be achieved, which is sufficient to disclose detailed gradients of molecular species within the stratum corneum (normal thickness about 15 μm) (11).

In this paper, the modification of urea's concentrations profile across the stratum corneum before and after reverse iontophoresis has been studied using confocal Raman microspectroscopy and the presence of a "skin reservoir" has been unequivocally demonstrated. Lactate concentration profile has also been found to be modified and has also been discussed.

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ABBREVIATIONS: Raman spectroscopy, RS; Infrared spectroscopy, IR; Natural moisturizing factor, NMF; Chronic kidney disease, CKD.

MATERIALS AND METHODS

Materials

Chemicals

Sodium chloride, L-histidine, Ag wire, AgCl, Pt wire, diacetylmonoxime, thiosemicarbazide, iron (III) chloride hexahydrate, ammonium hydroxide were obtained from Sigma-Aldrich (Schnelldorf, Germany). Urea, lithium sulfate monohydrate and sulphuric acid were supplied by VWR (Leuven, Belgium). All reagents were of analytical grade. Ultrapure water (conductivity <0.065 μS/cm) was used to prepare all aqueous solutions.

Methods

Extraction of Urea through the Skin by Reverse Iontophoresis

The clinical protocol was approved by the "Commission d'Éthique Biomédicale Hospitalo-Facultaire" of the "Cliniques Universitaires St Luc" (Brussels). Informed consent was obtained from all participants.

Eight healthy volunteers (A–H) and three patients (X–Z) with stage 5 chronic kidney disease (12) were involved in the experiment. One volunteer participated twice (A_1–A_2). For the healthy volunteers, two cylindrical plastic chambers (3 cm diameter) were adhered to the forearm with silicone grease (Dow Corning®) and medical tape (3M Foam Tape 9772L, 3M Healthcare, St Paul, MN, USA). For the three patients, glass cells (2 cm diameter) of a more practical and comfortable design were used. The cathodal chamber was filled with 10 mM L-histidine solution while the anodal compartment contained 133 mM NaCl (9 ml or 6 ml, in both compartments, depending on the type of cell). Ag/AgCl electrodes were inserted into the solutions and held at least at 5 mm from the skin surface by means of a plastic cover. A Phoresor II Auto (Model PM 850, Iomed Inc., Salt Lake City, UT) delivered a constant, direct current of 0.8 mA for 2 h during which samples were obtained at intervals of 10 min during the first 30 min, and at intervals of 10 or 30 min thereafter. At the end of each sampling interval, the current was stopped and the cathodal chamber solution was completely removed and refreshed. The samples were subsequently analyzed for urea and lactate, respectively by a validated colorimetric method (3,13) and by an enzymatic method (14).

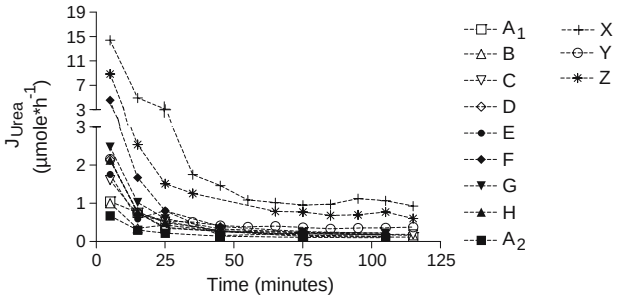

Fig. 1. Reverse iontophoretic extraction fluxes (J_{Urea}) as a function of time in eight healthy volunteers (*A–H*) and three patients with chronic kidney disease (*X–Z*). One of the volunteers participated twice (A_1–A_2). The hatched lines define the flux profile of each volunteer.

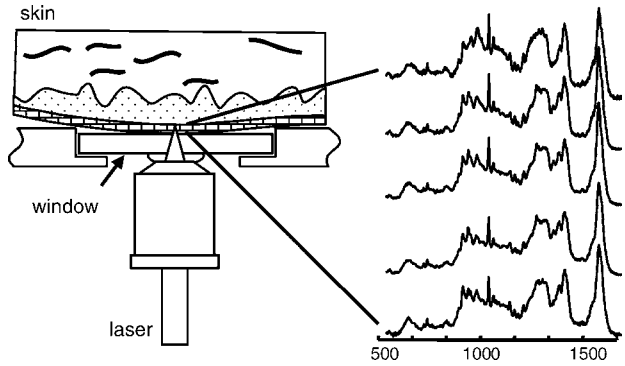

Fig. 2. Schematic representation of *in vivo* confocal Raman measurements on the skin. The microscope objective can be moved up and down to change the location of the laser focus in the skin. The Raman spectra reflect the local chemical composition at various depths in the stratum corneum.

Raman Spectroscopy

Raman measurements were performed using the model 3510 Skin Composition Analyzer (River Diagnostics, Rotterdam, The Netherlands). The instrument is optimized for rapid *in vivo* measurements on human skin and offers an axial spatial resolution of 5 μm. It comprises a high-performance dispersive spectrometer with 671 nm and 785 nm laser excitation and a confocal measurement stage. To realise Raman measurements, the arm of the volunteer was placed on a fused silica window mounted on the measurement stage. Laser light was focused in the skin with a microscope objective located under the window. The location of the laser focus relative to the skin surface could be accurately varied. By changing the focusing depth automatically, Raman spectra were recorded from the skin surface down to the viable epidermis in 2 μm depth increments in the fingerprint spectral region (400–1800 cm^{-1}) (Figs. 2 and 3). In this way, detailed Raman profiles were acquired across the stratum corneum. Measurement time was 10 s per profile point. To address the lateral variation in skin composition, about 10 profiles per area were measured.

All spectra were calibrated and corrected for instrument response using the built-in instrument control software of the model 3510 SCA (River Diagnostics). Relative urea and lactate contents were determined by classical least squares fitting, using the skin analysis toolbox (River Diagnostics). The fitting procedure is described in detail elsewhere (9). Briefly, a set of reference spectra of the major skin constituents is fitted to the Raman spectra obtained from the arm. The fit coefficients were normalized by the signal from keratin, which is the dominant dry mass fraction in the stratum corneum. The normalization step is necessary to correct for variations in the absolute Raman intensity, which decreases with distance from the skin surface. The procedure provides a measure of the local content of urea and lactate in the stratum corneum, relative to the amount of keratin. Because keratin was used as an internal quantification standard, the results were not influenced by any swelling of the skin due to the contact with the aqueous buffer.

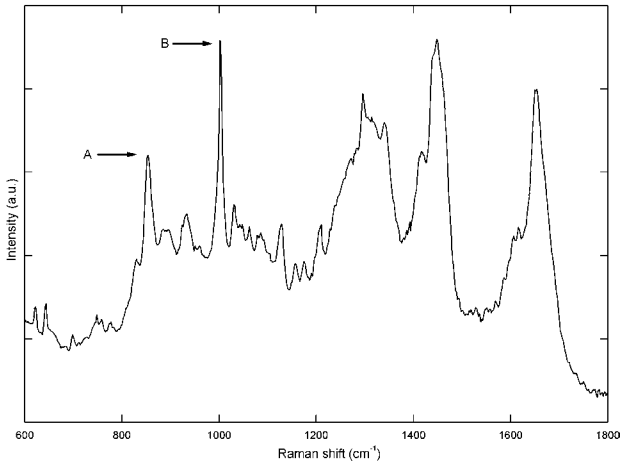

Fig. 3. *In vivo* Raman spectra of the untreated inner forearm, measured at 4 μm below the skin surface. Signal collection time: 10 s. The most prominent bands of lactate and urea, partially overlapping with Raman bands of keratin, are indicated with arrows. *A* lactate; *B* urea.

Skin Urea Reservoir Analysis by Raman Spectroscopy

Raman spectra were acquired before and after 30 min of iontophoresis. Four healthy volunteers were involved in the experiment. The cathodal and anodal chambers (glass cells, 2 cm diameter) contained the same electrolyte solutions as before (6 ml). A current of 0.8 mA was applied for 30 min. At the end of this period, the system was removed from the skin and a second series of Raman measurements was made. A control experiment was also performed on four subjects using the identical procedure but without current application.

Analytical Chemistry

The diacetylmonoxime colorimetric method used to assay urea was a validated adaptation (13) of a previously published method (15). Briefly, samples and standards (180 μL) were placed in 96-well plates and 15 μL of reagent A (0.336 mmol/L diacetylmonoxime and 0.0042 mmol/L thiosemicarbazide) as well as 48 μL of reagent B (30 mL concentrated sulphuric acid diluted to 53.5 mL with water together with 50 μL of 0.055 mmol/L ferric chloride solution), were added in each well. The plate was then mixed and placed at 85°C during 75 min. After heating, it was cooled with ice and red at 520 nm (3,13).

Interpretation of Iontophoresis Data and Statistics

Urea fluxes were calculated by dividing the amount collected in a sampling interval by the duration of that collection period (μmol/h). Data are expressed as mean +/− standard deviation. Statistical tests, non-linear regression analysis and graphical formatting were performed with Graph Pad Prism 4.0 software (San Diego, CA). A paired ratio *t* test was used to compare results pre- and post-treatment (16). MANOVA was used (1) to compare urea extraction fluxes from healthy volunteers with those from the patients, and (2) to evaluate the individual decrease in urea flux as a function of time. A matched pairs test was used to assess the stabilization of the urea extraction. The level of statistical significance was fixed at $p<0.05$.

RESULTS AND DISCUSSION

Reverse Iontophoresis of Urea in Volunteers

To examine the presence of a skin reservoir of urea *in vivo*, iontophoretically extracted fluxes by iontophoresis were measured over 2 h in healthy volunteers and in renally impaired patients. Urea extraction was higher at the beginning of current passage, but then decreased significantly ($p<0.05$) to achieve stable values after 2 h of iontophoresis (Fig. 1). This decrease was not related to variation in urea blood concentrations which remained relatively constant

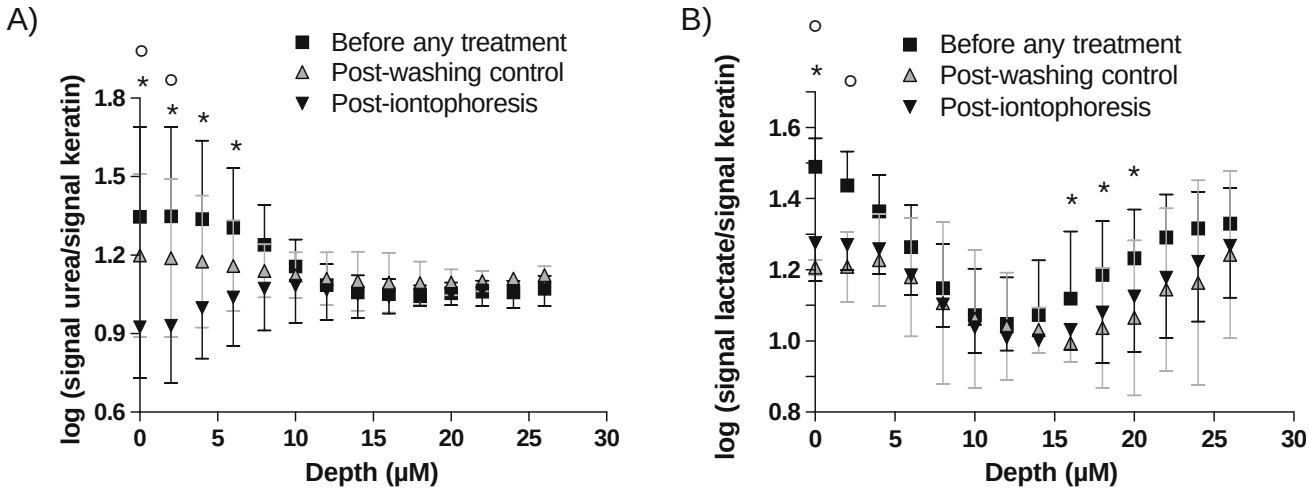

Fig. 4. **A** Logarithm of the normalized urea signal, relative to that from keratin, as a function of depth into the stratum corneum pre-treatment (*filled squares*), after 30 min of iontophoresis (*filled, inverted triangles*), and following 30 min of contact with aqueous buffer (no-current control) (*open triangles*). **B** Logarithm of the normalized lactate signal, relative to that from keratin, as a function of depth into the stratum corneum pre-treatment (*filled squares*), after 30 min of iontophoresis (*filled, inverted triangles*), and following 30 min of contact with aqueous buffer (no-current control) (*open triangles*). Significant differences: *Asterisks* $p<0.05$, iontophoresis vs. pre-treatment; *circles* $p<0.05$, no-current control vs. pre-treatment).

over this time-frame. Interestingly, the fluxes from patients with chronic kidney disease were significantly higher than those from the normal volunteers ($p<0.01$).

The initial samples collected after 10 min of iontophoresis contained 4–13 times more urea than those following an hour of current passage, strongly suggesting the existence of a skin 'depot' of the analyte. The subsequent decrease, with increasing time, to a constant level indicates the progressive depletion of this "reservoir" of the analyte. The stabilized extraction flux at longer times implies that these values are correlated with systemic (blood) concentrations as it has been shown previously for glucose and lithium, for example (17,18). The hypothesis of a urea reservoir in the skin is consistent with earlier *in vitro* experiments using pig skin (19): in that study, even when the subdermal compartment contained no urea, reverse iontophoresis resulted in significant, initial extraction of the compound to the cathode compartment on the skin surface (3). Similar observations, consistent with these results, have also been reported for glucose (18) and lactate (14).

The presence of a urea 'reservoir' in the skin is not unexpected, however. Natural moisturizing factor (NMF) accounts for nearly one-fifth of the total weight of the stratum corneum and contains ~7% urea (20). It follows that reverse iontophoresis, either *in vitro* or *in vivo*, initially extracts this 'local' supply of the analyte. After about 30 to 60 min, however, this depot will have been depleted and the levels extracted at this point are now correlated with those in the interstitial fluid of the viable skin (which are, in turn, in equilibrium with the blood). This is consistent with an earlier study, which showed that the initially iontophoretically-extracted urea fluxes were poorly correlated with the corresponding blood concentrations; in contrast, with increasing time of current passage, the correlation became highly significant and, by 2 h, was good enough to be predictive (21).

Observation of Urea Reservoir Depletion by Reverse Iontophoresis using Confocal Raman Spectroscopy

To confirm that reverse iontophoresis depleted the urea skin reservoir during the initial application of current, confocal Raman microspectroscopy was used to measure urea content in the skin *in vivo* as a function of depth. Depletion of a local depot of urea by iontophoresis would be expected to change its concentration gradient across the stratum corneum.

The Raman results obtained before and after application of current demonstrate that iontophoresis did indeed modify the distribution of urea at different depths in the skin (Fig. 4A). A Raman spectrum of the arm is shown in Fig. 3, with the arrows indicating the most prominent Raman bands from lactate (855 cm^{-1}) and urea (1003 cm^{-1}). After 30 min, a significant depletion of urea was observed, especially at the surface of the skin (0–6 μm) ($p<0.05$).

Unexpected results were found about the distribution of lactate which is also known to be a major constituent of NMF (12%) (20). Lactate distribution in the skin was modified at the surface but also at deeper locations of the skin (0;16–20 μm) ($p<0.05$) (Fig. 4B). Whereas the distribution of urea seemed to be mainly modified in surface, modifications also occurred deeper for lactate.

Control experiments showed that a 30-min exposure of the skin to aqueous buffer without current also modified the urea and lactate content of the stratum corneum in the most superficial layers (0–2 μm), but to an extent (and to a depth) that was significantly less than that induced when iontophoresis was applied (Fig. 4A and B).

We hypothesized that urea concentrations were depleted deeper in comparison with the "washing control" because of the extraction of urea by reverse iontophoresis. The depletion of the lactate concentrations deeper in the skin (16, 18 and 20 μm), was probably due in part to the repulsion of the negatively charged molecule with the negatively charged electrode. The "washing control" of lactate shows also diminished lactate concentrations at this depth, even not significant. The low activity of the arm during the iontophoresis session (30 min) and the second Raman measurement (30 min), and thus, the low production of lactic acid during this period, could also have contributed to the diminution of lactate concentration in the viable epidermis, in comparison with Raman measure (14). Urea and lactate are highly soluble in water and thus a dilution of these components in the buffer contained in the receptor chamber can explain the modification of urea and lactate concentration profiles at the upper layers of the skin.

CONCLUSION

Measurements of urea and lactate gradients in the skin using *in vivo* confocal Raman microspectroscopy were performed to complement *in vivo* monitoring of urea by reverse iontophoresis.

This novel approach demonstrates that urea skin concentrations profiles are modified after iontophoresis and these results are consistent with the hypothesis of a "urea skin reservoir" which should be depleted to obtain a relation between plasma urea concentrations and urea extracted fluxes. As the urea reservoir is unrelated with the blood concentration, an immediate monitoring of urea by reverse iontophoresis is not possible.

Nevertheless, as this reservoir is emptied using current application, longer periods of time have to be tested to take the "reservoir effect" factor apart. After its complete removal, a relation linking the urea blood concentration and their corresponding extracted fluxes can be obtained.

In vivo confocal Raman microspectroscopy is a novel technique, which provides a wealth of hitherto unavailable information about the distribution of endogenous and exogenous molecular components in the skin. It is currently the only method that enables rapid, non-invasive measurement of molecular gradients in the stratum corneum.

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