Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 1077–1082
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Non-invasive diagnosis and monitoring of chronic kidney disease by reverse iontophoresis of urea *in vivo*

Valentine Wascotte [a], Eric Rozet [b], Ana Salvaterra [a], Philippe Hubert [b], Michel Jadoul [a], Richard H. Guy [c], Véronique Préat [a,*]

[a] *Université Catholique de Louvain, Unité de Pharmacie Galénique, Brussels, Belgium*
[b] *Université de Liège, Laboratoire de Chimie Analytique, Liège, Belgium*
[c] *University of Bath, Department of Pharmacy and Pharmacology, Bath, United Kingdom*

Received 27 November 2007; accepted in revised form 14 February 2008
Available online 20 February 2008

Abstract

Background: Reverse iontophoresis uses a small current to extract molecules and ions through the skin. The aim of the study was to determine whether reverse iontophoresis of urea can be used (i) to diagnose and monitor non-invasively chronic kidney disease (CKD), and (ii) to track urea levels closely during a hemodialysis session.

Methods: A current of 0.8 mA was applied for 2 h in 10 healthy volunteers, in 9 patients with CKD, and in 10 patients undergoing hemodialysis. Urea fluxes extracted by reverse iontophoresis and urea concentrations in the blood were measured.

Results: Extracted urea fluxes discriminated healthy volunteers from patients with CKD within 90 min. A non-invasive measure of blood urea concentrations can be achieved after 120 min. A urea reservoir in the skin interferes with the extraction and a pre-hemodialysis "depletion" period is required. Mild and transient sensation and erythema induced by iontophoresis were significantly lower in the CKD group. Gelling the formulation of the iontophoresis reservoir gave similar results to those obtained when using a simple aqueous solution.

Conclusions: Reverse iontophoresis can be used to non-invasively diagnose individuals with CKD and to monitor urea concentrations in blood.

Keywords: Reverse iontophoresis; Urea; Non-invasive monitoring; Diagnosis; Chronic kidney disease

1. Introduction

Because of the kidney's potent adaptive mechanisms, renal failure only appears when more than half of the initial nephron mass is destroyed [1]. The clinical diagnosis of CKD is principally based on the measurement of the increased levels of urea and creatinine. Without this crucial monitoring information, it is impossible to delay the onset of end-stage kidney disease and the associated mortality and morbidity [2]. As the disease worsens, renal replacement therapy is required; alternatively, in hemodialysis (HD), knowledge of urea levels is crucial to provide information of the efficacy of the procedure, and the nutritional status of the patient, both of which are important outcome markers [3,4].

The challenges therefore are to improve the control of high-risk patients, such as those with diabetes and hypertension, to assess the evolution of the disease and to monitor during HD, while avoiding the obvious disadvantages of conventional blood sampling (including the need for qualified personnel, pain, and the risk of infection).

Reverse iontophoresis uses a low current (<0.5 mA/cm^2) to extract substances through the skin [5], and a device for the non-invasive monitoring of blood glucose (the Gluco-

* Corresponding author. Université Catholique de Louvain, Unit of Pharmaceutical Technology, Avenue Emmanuel Mounier, UCL 73 20, 1200 Bruxelles, Belgium. Tel.: +32 2 764 73 09; fax: +32 2 764 73 98.
E-mail address: veronique.preat@uclouvain.be (V. Préat).

0939-6411/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2008.02.012

Watch Biographer®), which uses this approach, has been commercialized recently [6]. As urea has already been shown to be extractable by reverse iontophoresis [7,8], we hypothesized that the method could be adapted for the diagnosis of CKD and the assessment of urea blood concentrations. This idea was first tested *in vitro*, where it was shown that reverse iontophoretically extracted urea fluxes were correlated with subdermal levels of the analyte, and were sensitive to time-dependent changes in these concentrations (so as to mimic a hemodialysis session); both diagnostic and monitoring applications may therefore be considered [9].

The aim of this study was to determine whether reverse iontophoresis of urea can be used (i) to diagnose and monitor non-invasively chronic renal failure, and (ii) to track urea levels during a hemodialysis session. In addition, to demonstrate the practicality of the approach, the development of simple gel formulations for the electrode reservoirs [10] was also investigated.

2. Materials and methods

2.1. Population

Healthy subjects (age range: 24–47 years; 4 males, 6 females) (with no history of renal or skin disease) and patients with CKD (with no history of skin disease) participated in the study. Patients were not receiving corticosteroids and were free of hepatitis B, C, and HIV. Nine patients with stage 5 CKD [11] (age range: 42–84 years; 7 males, 2 females) were involved in the diagnostic study, while 10 patients were studied during a HD session (age range: 60–85 years; 8 males, 2 females). All protocols were approved by the *Commission d'Éthique Biomédicale Hospitalo-Facultaire* of the Cliniques Universitaires Saint-Luc, Brussels, and informed consent was obtained from each of the participants.

2.2. Materials

Urea (>99.5%), lithium sulphate monohydrate, and sulphuric acid (>95%) were supplied by VWR (Leuven, Belgium). NaCl, KCl, L-histidine, Ag wire (99.9%), AgCl (99%), Pt (99.9%), diacetylmonoxime (>98% pure), thiosemicarbazide, iron (III) chloride hexahydrate (>98%), and Pluronic F-127 were purchased from Sigma (Schnelldorf, Germany). All reagents were of analytical grade. Na^+ and K^+ calibration standards and IL Test® were from Instrumentation Laboratory (Milan, Italy). Ultrapure water (conductivity less than 0.065 μS/cm) was used to prepare all solutions.

2.3. Reverse iontophoresis of urea to diagnose and monitor CKD

Two cylindrical glass chambers (2 cm diameter) were firmly attached to the volunteer's forearm with silicone grease (Dow Corning, Midland, MI) and medical tape (3M Foam Tape 9772L, 3M Healthcare, St. Paul, MN, USA). The cathodal chamber was filled with 6 mL of a 10 mmol/L solution of L-histidine, while the anodal compartment contained an equal volume of 10 mmol/L L-histidine solution in 133 mmol/L NaCl [9]. Ag/AgCl electrodes were inserted into the solutions and held at least at 5 mm from the skin surface by means of a specially designed 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 to the electrodes. At intervals of 30 min, the current was stopped and the cathodal chamber solution was completely removed for analysis and replaced with a fresh buffer. Between 10 and 30 min after the end of the experiment, a blood sample was acquired from the volunteer.

To evaluate the ability of the iontophoretic technique to adequately predict urea blood concentrations, the results from 7 healthy volunteers and from 6 patients (not tested during HD) were used to generate a linear relationship between urea blood concentration and urea flux. Then, for 3 other healthy volunteers and 3 other patients, urea blood levels were predicted from the corresponding extraction fluxes using this relationship and were subsequently compared to the experimentally measured values.

2.4. Reverse iontophoresis of urea to follow HD

As described above, two electrode chambers were affixed to the skin and filled with the same electrolyte solutions [9]. A current of 0.8 mA was applied for 30 min before the HD session began. Samples of the cathode solution were then taken every 10 min over the period of current passage. Blood samples were withdrawn at the start of HD, during the HD session, and at the end of current application.

2.5. Reverse iontophoresis using a gel formulation

A Pluronic F-127 gel was prepared as described previously [12]. Briefly, the polymer was dispersed in a cold solution of L-histidine (10 mmol/L) with or without NaCl (200 mmol/L) and was continuously stirred at 4 °C until it was clear. The concentration of Pluronic F-127 was 22% w/w to produce a thermoreversible gel, liquid at 4 °C and gel at room temperature. The cold solutions were therefore easily transferred to the chambers on the skin surface where gelification subsequently occurred *in situ*. Reverse iontophoretic urea extraction to the cathode was then compared with gelled and ungelled formulations (applied on opposite arms). Separate power supplies were used for the two pairs of electrode chambers. A current of 0.8 mA was applied for 1 h with samples obtained at 30 and 60 min.

2.6. Local effects of iontophoresis on healthy volunteers and patients

The degree of sensation felt during reverse iontophoresis was evaluated by the adaptation of the *Questionnaire Doul-*

eur de Saint Antoine vocabulary test in both healthy volunteers and patients: the subjects selected from a list of descriptive adjectives those which best characterized the sensation experienced. The intensity of the sensation was also evaluated on a numeric scale from 0 to 10, where 0 represents the absence of sensation and 10 the maximum sensation imaginable. The erythema observed at the end of the current application was scored from 0 to 4 as follows: 0 = no trace of redness on the skin; 1 = weak redness; 2 = a marked redness with up to 3 or 4 punctate marks; 3 = strong redness with or without punctate marks; 4 = evidence of a skin burn.

2.7. Analytical chemistry

The reverse iontophoretically extracted samples were subsequently analysed for urea by a validated colorimetric method [9,13]. Briefly, samples and standards (180 μL) were placed in 96-well plates with 15 μL of reagent A, which contained diacetylmonoxime at 34 mg/mL and thiosemicarbazide at 95 mg/mL and 48 μL of reagent B (comprising 30 mL of concentrated sulphuric acid diluted to 53.5 mL with distilled water and 50 μL of ferric chloride at 15 mg/mL). The samples were warmed to 85 °C for 75 min and measured at 520 nm.

As this analytical technique could not be directly applied to the gel samples, a dialysis method was used to recover the analytes. To relate the concentration recovered to that present in the gel, a calibration curve was established using known urea concentrations in the gel [10].

Side-by-side glass cells (2 mL compartment volume and 0.5 cm^2 dialysis surface) were designed. A cellulose dialysis membrane (Spectra/Por® 7, MWCO 3500, Spectrum® Laboratories, Inc., Rancho Dominguez, CA) was clamped between the two compartments with the assistance of two silicone rings. 1.5 mL of a Pluronic solution (22% w/w and 10 mmol/L L-histidine) and 1.5 mL of a L-histidine solution (10 mmol/L) were placed into the donor and receiver compartments, respectively. Known amounts of urea and Na^+ were introduced into the gel to calibrate the efficiency of the process. To ensure complete extraction, the dialysis continued for 15 h at 4 °C (with continuous stirring of both compartments).

2.8. Data analysis and statistics

Urea fluxes were calculated by dividing the amount collected during a sampling interval by the duration of that collection period and expressed in μmol/h. Data are expressed as means ± standard deviation (SD). The significance of linear regressions was assessed by ANOVA at the level of $p < 0.05$. 95% prediction intervals were determined from the data and used to delimit "decision regions" in which normal and renally impaired individuals can be identified with a risk of false positive and false negative of maximum 5% error (or less) of false positives or false negatives [14].

A Wilcoxon test was used to compare levels of sensation, and a χ^2-test was employed for erythema. The recoveries from the gel were obtained by regression curves, covering the urea and sodium concentration ranges collected in the gel.

Data manipulation, linear, and non-linear regressions used Graph Pad Prism V 4.0 (Graph Pad Software, Inc., San Diego, CA); the testing of the distributions and the evaluation of prediction intervals together with decision regions were made with JMP V 6.0 (SAS Institute, Cary, NC).

3. Results and discussion

3.1. Time-dependence of urea extraction flux

The extracted fluxes of urea decreased as a function of time both in healthy volunteers (Fig. 1A) and in patients with stage 5 CKD (Fig. 1B). This behaviour is due to the presence of a urea reservoir in the skin, and is consistent with previous *in vitro* experiments [9]; in that work, even when the subdermal compartment contained no urea, reverse iontophoresis was able to extract significant amounts to the cathodal compartment on the skin surface. The results are also in agreement with a recent confocal Raman spectroscopy study which clearly located the presence of a urea reservoir in the stratum corneum [15], due (at least, in part) to the contribution of this small non-elec-

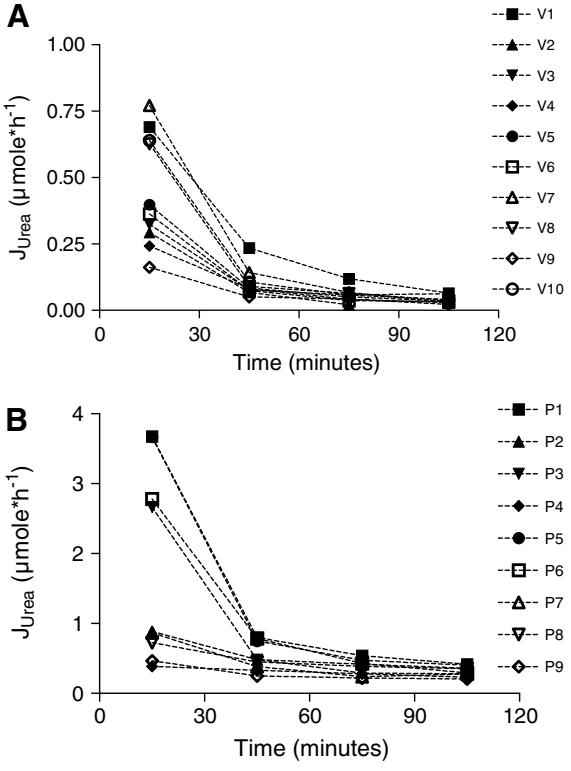

Fig. 1. Reverse iontophoretic extraction fluxes of urea as a function of time in (A) 10 healthy volunteers, (B) 9 patients with stage 5 CKD. Note that the ordinate scale is smaller in (A) than in (B).

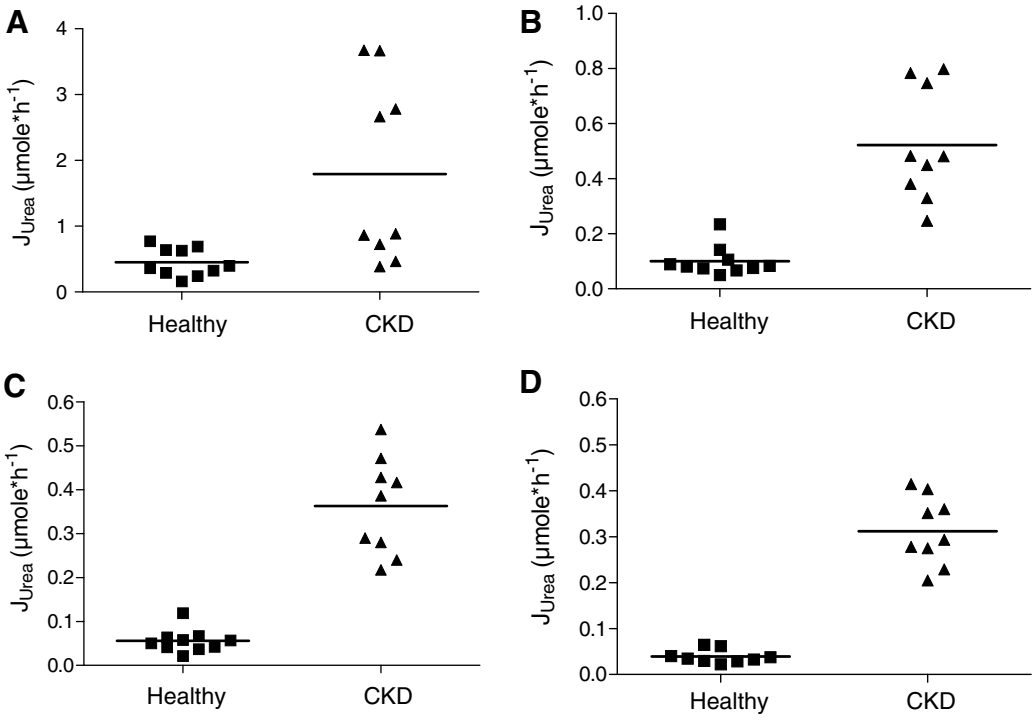

Fig. 2. Reverse iontophoretic extraction fluxes of urea in healthy volunteers and in patients with CKD after (A) 30; (B) 60; (C) 90; and (D) 120 min of current passage. Note that the ordinate scale decreases with increasing time of iontophoresis.

trolyte to the skin's natural moisturizing factor, reported to contain ~7% w/w urea [16].

3.2. Non-invasive diagnosis of chronic kidney disease by reverse iontophoresis

Fig. 2 compares the reverse iontophoretic urea extraction fluxes in healthy volunteers and in CKD patients as a function of the time of current passage. As the duration of iontophoresis increases, the distinction between normal subjects and those with renal impairment becomes progressively clearer.

Computation of the 95% prediction intervals for the two populations reveals that 90 min of iontophoresis is sufficient to obtain an acceptable separation between the groups, indicating the potential for a useful diagnostic tool (Table 1).

Table 1
95% "prediction intervals" and corresponding "decision regions" for reverse iontophoretic urea extraction fluxes (µmol/h) in healthy and CKD subjects as a function of time of current passage

Time of iontophoresis (min)	95% prediction interval		"Decision region"	
	Healthy	CKD	Healthy	CKD
30	0–0.95	0–5.1	nc*	nc
60	0–0.23	0.04–1.01	nc	nc
90	0–0.12	0.10–0.63	<0.11	>0.15
120	0.015–0.073	0.14–0.49	<0.067	>0.17

* nc, not calculable due to the large overlap of results from healthy and CKD subjects.

From the derived prediction intervals, it is then possible to define "decision regions" [14] in which the reverse iontophoretic flux of urea will predict with a maximum risk of 5% inadequate decision whether a subject is healthy or is suffering from severe CKD. These values are also reported in Table 1. Clearly, subjects whose values fall in between the "decision regions" would be those requiring additional monitoring and follow-up.

3.3. Non-invasive monitoring of urea blood concentrations by reverse iontophoresis

The urea fluxes presented in Fig. 2 (J_{urea} in µmol/h) were next correlated with the corresponding urea blood levels (C_{urea} in mg/dL). A linear relationship was apparent after 60 min ($r > 0.7$), and became more significant with increasing time of current passage (Fig. 3).

To examine whether this correlation could be used to predict urea concentrations in the blood from the iontophoretically extracted flux of the analyte, the subjects were arbitrarily divided into a "training group", which included 6 patients with stage 5 CKD and 7 healthy volunteers, and which was used to define the linear relationship, and a "test group" of 3 patients and 3 volunteers. As a period of 60 min is not sufficient to separate healthy volunteers from CKD patients, only the 90 and 120 min data were considered.

The linear relationships from the "training group" were

$$90\ \text{min}: \quad J_{urea} = (1.92 \times 10^{-3}) * C_{urea} + 0.023 \qquad r^2 = 0.94 \quad (1)$$

$$120\ \text{min}: \quad J_{urea} = (1.56 \times 10^{-3}) * C_{urea} + 0.017 \qquad r^2 = 0.93 \quad (2)$$

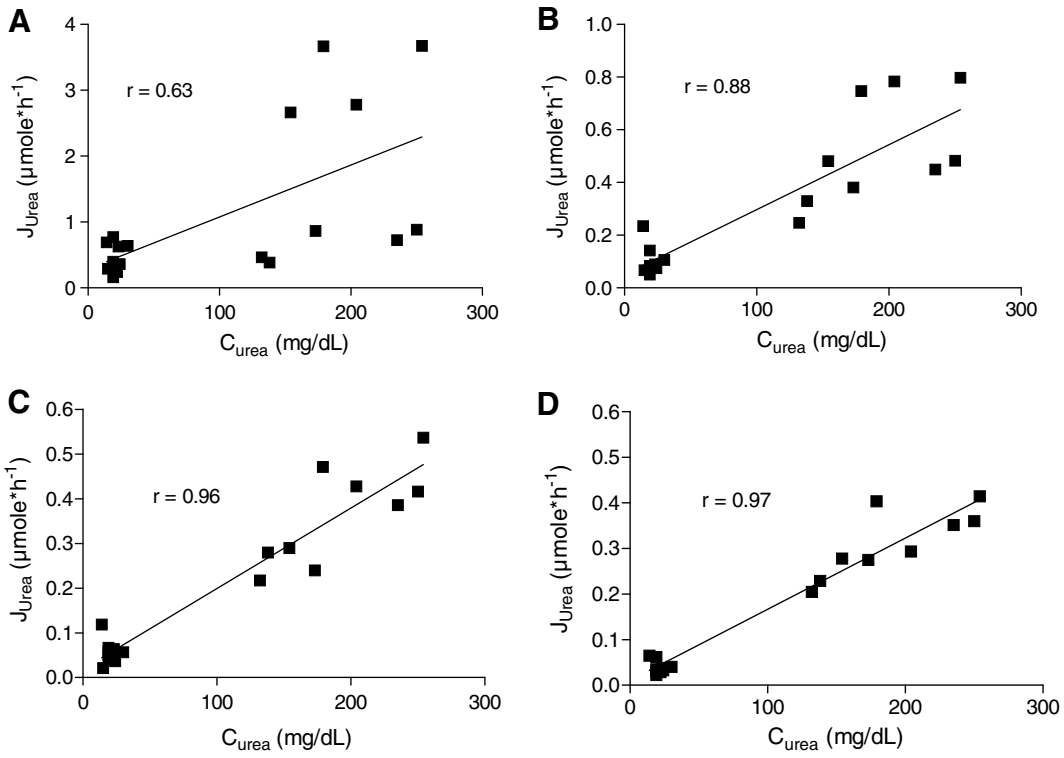

Fig. 3. Reverse iontophoretic extraction fluxes of urea after (A) 30; (B) 60; (C) 90; and (D) 120 min of current passage plotted as a function of the corresponding blood concentrations measured in the same volunteers 10–30 min after the end of the experiment.

These correlations were then used to predict C_{urea} from the measured J_{urea} at 90 and 120 min in the subjects of the "test group". These predictions are compared to the experimentally measured blood levels in Fig. 4 and show good agreement.

Whether this approach might be developed into a useful practical tool remains to be seen. Obviously, the presence of the skin's urea reservoir, which must be first cleared before systemic information can be obtained, prolongs (at least, for now) the time of iontophoresis necessary to an unacceptable duration.

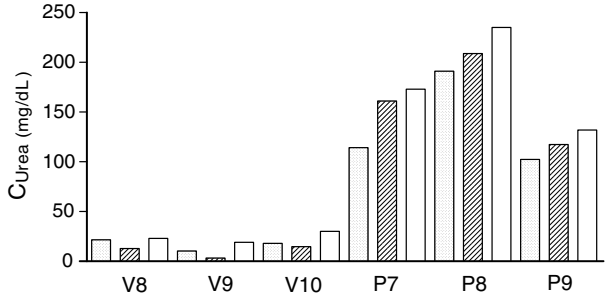

Fig. 4. Comparison between predicted (filled and hatched bars) and experimentally measured (open bars) urea blood concentrations in 3 healthy volunteers (V8, V9, V10) and 3 CKD patients (P7, P8, P9). The predicted values were obtained from the reverse iontophoretically measured urea extraction fluxes in these subjects and the relationships (Eqs. (1) and (2), filled and hatched bars, respectively) derived from an independent subset of subjects (comprising V1–V7 and P1–P6).

3.4. Non-invasive monitoring of urea during hemodialysis

As observed *in vitro* [9], reverse iontophoretic extraction of urea during HD tracked the exponential decay of the analyte in the blood (Fig. 5). However, the significant contribution of the urea reservoir to the amounts of analyte extracted means that it is difficult to distinguish the fluxes obtained in CKD patients during and apart from HD. Again, therefore, refinement of the approach is needed to

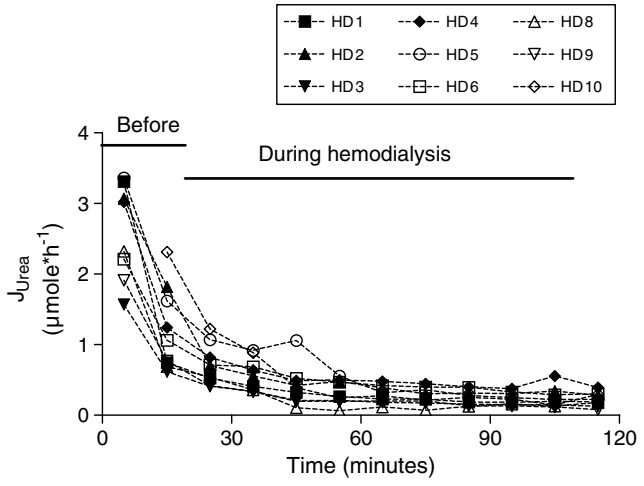

Fig. 5. Reverse iontophoretic extraction fluxes of urea as a function of time in 10 patients for 30 min prior to hemodialysis (HD) and during a subsequent 90-min HD session.

render the method practically useful for monitoring the progress of a HD session.

3.5. Local effects of iontophoresis in healthy volunteers and in patients with CKD

As previously reported in the literature [17,18], only very mild sensation, most often described as "tingling", was experienced when iontophoresis was applied. Typically, the sensation was more pronounced at the anode and diminished rapidly with time. Sensation was most noticed at the beginning and just after the termination of current flow. CKD patients reported less sensation (median response = 1 on a 10-point scale) than normal subjects (median response = 1.5) ($p = 0.0043$).

A very mild and transient erythema was observed in most volunteers. In a small number, a few punctate marks were also observed at the end of the experiment. Erythema scores were significantly lower ($p = 0.006$) in patients (five level 0 and four level 1) than in healthy volunteers (in 50% of which an erythema score of 2 was observed).

3.6. Gel formulation

A linear relation ($Y = 0.92X - 3.32$; $r^2 = 0.95$) between the urea concentrations measured in the liquid and gel cathodal compartments after 30 and 60 min of reverse iontophoresis was found combining the results of 2 patients and 3 healthy volunteers.

The extraction of urea was as efficient as that into a solution, suggesting that this necessary component of a practical device is technologically feasible.

4. Conclusion

This study demonstrates that the non-invasive, reverse iontophoretic monitoring of urea across the skin *in vivo* is feasible, and that applications in the diagnosis and monitoring of renal impairment may be envisaged. Although technologically possible, however, there remain significant challenges before the development of a useful and practical device is considered. The presence of a urea reservoir in the outer skin layers (due to its contribution to the barrier's natural moisturizing factor), in particular, confounds the specificity of the approach for tracking systemic urea levels and requires, for the moment, that the iontophoretic current be passed for unacceptably long periods of time before useful information can be obtained.

Acknowledgments

This work was funded by FRSM and FNRS. Valentine Wascotte thanks the nephrology staff of the Cliniques Universitaires Saint-Luc, Brussels, for their invaluable contribution. The authors gratefully acknowledge the statistical help and guidance of Professor Jean Cumps (Université Catholique de Louvain, Belgium).

References

[1] B.M. Brenner, T.H. Hostetter, S.C. Hebert, Anomalies de la fonction rénale, in: T.R. Harrison (Ed.), Principes De Médecine Interne, Médecine-Sciences Flammarion, Paris, 1996, pp. 1138–1144.
[2] N. Dombros, M. Dratwa, M. Feriani, R. Gokal, O. Heimburger, R. Krediet, J. Plum, A. Rodrigues, R. Selgas, D. Struijk, C. Verger, European best practice guidelines for peritoneal dialysis. 2. The initiation of dialysis, Nephrol. Dial. Transplant. 20 (Suppl. 9) (2005) ix3–ix7.
[3] N. Man, M. Touam, P. Jungers, Dialyse adéquate et équilibre nutritionnel, L'Hémodialyse De Suppléance, Flammarion, Médecine-Sciences, Paris, 2003 (pp. 58–71).
[4] F. Locatelli, U. Buoncristiani, B. Canaud, H. Kohler, T. Petitclerc, P. Zucchelli, Haemodialysis with on-line monitoring equipment: tools or toys? Nephrol. Dial. Transplant. 20 (2005) 22–33.
[5] B. Leboulanger, R.H. Guy, M.B. Delgado-Charro, Reverse iontophoresis for non-invasive transdermal monitoring, Physiol. Meas. 25 (2004) R35–R50.
[6] M.J. Tierney, J.A. Tamada, R.O. Potts, R.C. Eastman, K. Pitzer, N.R. Ackerman, S.J. Fermi, The GlucoWatch biographer: a frequent automatic and noninvasive glucose monitor, Ann. Med. 32 (2000) 632–641.
[7] A. Sieg, R.H. Guy, M.B. Delgado-Charro, Simultaneous extraction of urea and glucose by reverse iontophoresis in vivo, Pharm. Res. 21 (2004) 1805–1810.
[8] I.T. Degim, S. Ilbasmis, R. Dundaroz, Y. Oguz, Reverse iontophoresis: a non-invasive technique for measuring blood urea level, Pediatr. Nephrol. 18 (2003) 1032–1037.
[9] V. Wascotte, M.B. Delgado-Charro, E. Rozet, P. Wallemacq, P. Hubert, R.H. Guy, V. Préat, Monitoring of urea and potassium by reverse iontophoresis in vitro, Pharm. Res. 24 (2007) 1131–1137.
[10] V. Wascotte, B. Leboulanger, R.H. Guy, M. Begona Delgado-Charro, Reverse iontophoresis of lithium: electrode formulation using a thermoreversible polymer, Eur. J. Pharm. Biopharm. 59 (2005) 237–240.
[11] A.S. Levey, K.U. Eckardt, Y. Tsukamoto, A. Levin, J. Coresh, J. Rossert, Z.D. De, T.H. Hostetter, N. Lameire, G. Eknoyan, Definition and classification of chronic kidney disease: a position statement from Kidney Disease: Improving Global Outcomes (KDIGO), Kidney Int. 67 (2005) 2089–2100.
[12] E.J. Ricci, M.V. Bentley, M. Farah, R.E. Bretas, J.M. Marchetti, Rheological characterization of Poloxamer 407 lidocaine hydrochloride gels, Eur. J. Pharm. Sci. 17 (2002) 161–167.
[13] E. Rozet, V. Wascotte, N. Lecouturier, V. Preat, W. Dewe, B. Boulanger, P. Hubert, Improvement of the decision efficiency of the accuracy profile by means of a desirability function for analytical methods validation. Application to a diacetyl-monoxime colorimetric assay used for the determination of urea in transdermal iontophoretic extracts, Anal. Chim. Acta 591 (2007) 239–247.
[14] E. Trullols, I. Ruisanchez, F.X. Rius, J. Huguet, Validation of qualitative methods of analysis that use control samples, Trends Anal. Chem. 24 (2005) 516–524.
[15] V. Wascotte, P. Caspers, J. de Sterke, M. Jadoul, R.H. Guy, V. Préat, Assessment of the "skin reservoir" of urea by confocal Raman microspectroscopy and reverse iontophoresis in vivo, Pharm. Res. 24 (2007) 1897–1901.
[16] J.P. Marty, NMF and cosmetology of cutaneous hydration, Ann. Dermatol. Venereol. 129 (2002) 131–136.
[17] R.M. Brand, P. Singh, E. Aspecarranza, H.I. Maibach, R.H. Guy, Acute effects of iontophoresis on human skin in vivo: cutaneous blood flow and transepidermal water loss measurements, Eur. J. Pharm. Biopharm. 43 (1997) 133–138.
[18] R. van der Geest, D.A.R. Elshove, M. Danhof, A.P.M. Lavrijsen, H.E. Boddé, Non-invasive assessment of skin barrier integrity and skin irritation following iontophoretic current application in humans, J. Control. Release 41 (1996) 205–213.