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Research Paper

Monitoring of Urea and Potassium by Reverse Iontophoresis *In Vitro*

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Purpose. Reverse iontophoresis is an alternative to blood sampling for the monitoring of endogenous molecules. Here, the potential of the technique to measure urea and potassium levels non-invasively, and to track their concentrations during hemodialysis, has been examined.
Materials and Methods. *In vitro* experiments were performed to test (a) a series of subdermal urea and potassium concentrations typical of the pathophysiologic range, and (b) a decreasing profile of urea and potassium subdermal concentrations to mimic those which are observed during hemodialysis.
Results. (a) After 60–120 min of iontophoresis, linear relationships ($p<0.05$) were established between both urea and potassium fluxes and their respective subdermal concentrations. The determination coefficients were above 0.9 after 1 h of current passage using sodium as an internal standard. (b) Reverse iontophoretic fluxes of urea and K^+ closely paralleled the decay of the respective concentrations in the subdermal compartment, as would occur during a hemodialysis session.
Conclusions. These *in vitro* experiments demonstrate that urea and potassium can be quantitatively and proportionately extracted by reverse iontophoresis, even when the subdermal concentrations of the analytes are varying with time. These results suggest the non-invasive monitoring of urea and potassium to diagnose renal failure and during hemodialysis is feasible, and that *in vivo* measurements are warranted.

KEY WORDS: non-invasive monitoring; potassium; renal failure; reverse iontophoresis; urea.

INTRODUCTION

The development of non-invasive, or minimally invasive, technologies for patient monitoring is a subject of considerable research activity. Reverse iontophoresis is one such approach and uses a small electric current to painlessly enhance the transport of charged ions and polar, neutral compounds across the skin (1,2). The singular advantage of iontophoresis is that the level and duration of current applied directly controls molecular transport, irrespective of whether the principal mechanism involved is electromigration or electroosmosis (2–5). In the former case, the flux (J) of an ion (a) is given directly by Faraday's law:

$$J_i = \frac{t_i \cdot I}{F \cdot z_i} \tag{1}$$

where I is the current, F is Faraday's constant, and z_i and t_i are charge and transport number, respectively, of the ion. The transport number is the fraction of the total current transported across the skin by a specific species (thus, $t_i=I_i/I$) and reflects that the ions in the iontophoretic circuit (both exogenous and endogenous) compete with one another to carry the charge delivered by the power source. The degree to which an ion is able to transport the current depends upon its concentration, mobility and charge relative to the values of the corresponding parameters for all other ions in the system (6). In the latter case, a convective flow of solvent results from the imposition of an electric field across the skin which, at physiological pH, has a net negative charge (7). The direction of electroosmotic (EO) flow follows that of the ions of opposite charge to the membrane; that is, in this case, from the anode to the cathode. The resulting flux ($J_{EO,i}$) of the neutral substance (5) is then:

$$J_{EO,i} = \nu \cdot C_i \tag{2}$$

where C_i is the concentration of the species and v is the net volume flow across the skin. The latter depends, of course, on

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ABBREVIATIONS: BUN, Blood Urea Nitrogen; I, current; IS, internal standard; J, flux; r^2, determination coefficient; T, transport number.

the charge on the membrane and the intensity of current applied (2).

The symmetry of iontophoresis means that it can be applied to both drug delivery and to clinical monitoring. While the former can be optimized for the specific drug concerned, the greatest challenge in "reverse" mode is the availability of a specific and highly sensitive analytical method with which to detect the levels of the compounds targeted for extraction. Nonetheless, this is an obtainable objective and has led to the regulatory approval and commercialization of the GlucoWatch Biographer® (Anima Corp., West Chester, PA), a device which uses reverse iontophoresis to extract and monitor (following a single calibration with a blood sample) blood glucose levels in diabetics more or less continuously over a 13-h period (8). Other analytes have also been considered, including lithium, which has been successfully monitored in bipolar disorder patients receiving this drug (9), and urea, whose detection *in vivo* has been demonstrated and proposed in patients with renal disease (10).

This use of reverse iontophoresis to monitor kidney function is the focus of the present study. It is known that urea in the plasma increases with the degree of renal failure and accumulates in numerous kidney diseases (11); blood urea nitrogen (BUN) measurement is a routine test used primarily to evaluate kidney function. A non-invasive method to monitor urea would be valuable for patients at high-risk of developing renal problems, such as those suffering diabetes and hypertension. Equally, for individuals with compromised renal function who are regularly undergoing hemodialysis, reliable pre- and post-treatment measurements, as well as *in situ* monitoring, during dialysis, would be beneficial (11), reducing the risk of infection and anaemia (12,13). The potential to follow urea essentially continuously, furthermore, may reduce errors from urea rebound at the end of dialysis (14,15), allow systematic observations of the patient's status to be made (to avoid empiricism (16)), and to get accurate post-dialysis BUN (15,17,18).

Potassium is a crucial marker of cardiac function, which can be adversely affected by either hyper- or hypokalemia. In end-stage renal disease, K^+ can accumulate in the body because the kidneys fail to eliminate it (19). On the other hand, dialysis can result in very low K^+ levels which lead to cardiac dysfunction (20). Moreover, despite often severe dietary controls, nearly a quarter of all emergency dialyses are performed to correct hyper-kalemia, a condition that results in significant mortality (21). Conventional blood sampling for K^+ monitoring is not without problems due to the potential for hemolysis which results in artefactually high results (22,23); once more, therefore, the advantage of non-invasive, reverse iontophoretic monitoring seems clear.

The concurrent monitoring of urea and K^+ by reverse iontophoresis is an ultimate objective of this research.The two analytes pose different analytical challenges and their extraction across the skin exploits different mechanisms — electromosis for the uncharged urea, electromigration for the cationic K^+. For both substances, however, as for glucose when monitored with the GlucoWatch®, calibration with a blood sample is needed to relate the extracted amounts to the systemic concentration (24). An alternative strategy involves the concomitant extraction and detection of a so-called "internal standard" (IS) (25,26), the concentration of which (C_{IS}) in the blood is known and fixed. It has been shown, both *in vitro* and *in vivo*, that the ratio of the extraction flux of the analyte of interest (J_A) to that of the IS (J_{IS}) is proportional to the ratio of their subdermal concentrations:

$$\frac{J_A}{J_{IS}} = K \cdot \frac{C_A}{C_{IS}} \tag{3}$$

where K is a constant. Further, since C_{IS} is known and fixed, it follows that C_A can be deduced from a measurement of the ratio of the iontophoretic extraction fluxes:

$$\frac{J_A}{J_{IS}} = K' C_A \tag{4}$$

where $K' = K/C_{IS}$. The sodium ion has been proposed and shown to be a useful IS in that its plasma level does not vary by more than ±10% (25,27) and, relative to urea and K^+ during hemodialysis, the concentration of Na^+ is quite constant (28).

The first steps in developing a non-invasive tool for monitoring urea and K^+ in renal failure patients are described in this paper. Before the approach can be evaluated *in vivo*, though, technical feasibility must be demonstrated under carefully controlled *in vitro* conditions. Using a well-established experimental model, therefore, the linearity of urea and K^+ extraction with their respective subdermal concentrations has first been established. At the same time, the potential utility of Na^+ as an IS has been explored. Next, the decaying concentrations of urea and K^+ during a dialysis procedure have been simulated using typical changes in blood concentrations as a function of time (29) and, once again, the value of the IS approach has been considered. Finally, as patients with renal failure are subject to acidosis, and because the net charge on the skin is influenced by the pH of the media on either side of the membrane (4), the extraction of urea and K^+ at two different pH values overestimating the "delta" typically seen in acidosis was determined.

MATERIAL AND METHODS

Materials

Urea (>99.5%), lithium sulphate monohydrate and sulphuric acid (>95%) were supplied by VWR (Leuven, Belgium). NaCl, KCl, L-Histidine, HEPES, Ag wire 99.9%, AgCl 99%, Pt 99.9%, dicetylmonoxime (>98% pure), thiosemicarbazide, iron (III) chloride hexahydrate (>98%), ammonium hydroxide, and albumin from human serum (96–99%) were purchased from Sigma (Schnelldorf, Germany). All reagents were of analytical grade. Na^+ and K^+ calibration standard, IL Test®, was from Instrumentation Laboratory (Milan, Italy). Ultrapure water (conductivity < 0.065 μS/cm) was used to prepare the different solutions.

Skin Preparation

Porcine ears were obtained fresh from the experimental surgery department (Université Catholique de Louvain) and were cleaned under cold running water. The whole skin was removed carefully from the outer region of the ear and separated from the underlying cartilage with a scalpel. The skin was then dermatomed to an average thickness of 300 μm (Robbins Instruments, Chatham, USA) and the samples were wrapped individually in Parafilm® and maintained at −20°C for no longer than 3 months. All experiments were performed using skin from at least three different pigs (4).

Reverse Iontophoresis Experiments

In a first series of experiments, the reverse iontophoretic extraction of urea from the subdermal compartment of a three-chamber diffusion cell (30) was measured. In this *in vitro* model, a central, subdermal compartment is separated from the anode and cathode chambers by two pieces of excised porcine skin, oriented so that the surfaces of the tissue (area ~0.64 cm^2) faced into the electrode compartments. The cathode electrolyte was 2 ml of 10 mmol/l L-histidine (pH 7.47), a skin-compatible solution which was adequate to conduct the current (9); the anode solution of the same volume consisted of 133 mmol/l NaCl in 25 mmol/l HEPES buffer at pH 7.40. The subdermal chamber (0.54 ml) which was thermostatted at 37°C, contained the same physiological buffer together with urea which had been added at a level (2.5, 12.5, 25, 37.5 and 50 mmol/l) spanning the blood concentrations observed in patients with impaired renal function. A control experiment was also performed in which no exogenous urea was introduced into the subdermal solution. Iontophoresis was achieved by passing a constant current of 0.2 mA (0.31 mA/cm^2) for 6 h from a power supply (Moor Instruments, Axminster, England) via silver–silver chloride electrodes, which had been inserted into the anode and cathode chambers. To maintain the subdermal urea concentration, the compartment was perfused continuously with the appropriate stock solution using a peristaltic pump operating at 6.5 $ml.min^{-1}$. The electrode solutions were magnetically stirred throughout the experiment except when the cathode solution was sampled at 10, 20, 30, 60, 90, 120, 150, 210, 270 and 360 min post-initiation of current flow. At these times, the current was stopped and the entire contents of the cathode chamber were removed for analysis and replaced with fresh 10 mmol/l L-histidine solution.

In the second set of experiments, the extraction of potassium from the subdermal solution was determined. In this case, a simpler, two-compartment (side-by-side) diffusion cell was used, and a single piece of porcine skin (area~0.64 cm^2) separated the subdermal chamber, which also served as the anode compartment, from the cathodal solution contacting the outer surface of the membrane. The cathodal solution again comprised 10 mmol/l L-histidine; the subdermal/anodal chamber was filled with the same HEPES-buffered saline but to which, on this occasion, potassium chloride had been added at a physiologically relevant level (3, 4, 5 or 6 mmol/l). While the current employed was higher (0.4 mA, 0.62 mA/cm^2), the sampling times and procedure were identical to those used in the urea experiments.

A third experiment briefly examined whether acidosis of the subdermal solution had a significant effect on urea and potassium extraction. This study was performed at a single current of 0.4 mA and only one composition of the subdermal phase: 25 mmol/l urea and 4 mmol/l KCl in 133 mmol/l NaCl and 25 mmol/l HEPES buffered to pH 7.00 or 7.40 by titration with concentrated NH_4OH. The experiment was carried out in the side-by-side diffusion cells and otherwise followed the same procedures as the K^+ experiments described above.

Subsequently, reverse iontophoresis was used to monitor a simulation of the decrease in urea and K^+ concentrations observed during hemodialysis. The side-by-side, two-chamber diffusion cell was again employed with identical background electrolyte and constant current conditions as before. The initial urea and K^+ concentrations in the subdermal (anodal) compartment were 30 and 4.7 mmol/l, respectively. Iontophoresis was first performed for two periods of 30 min with this initial subdermal solution. At this point, it was considered that the reverse iontophoretic extraction rate, illustrative of the typical situation at the start of a hemodialysis session, had been established. The urea and K^+ concentrations were then decreased in a stepwise fashion over the next 4 h; the urea levels investigated were 25.8, 19.2, 14.2 and 10.4 mmol/l, while the corresponding K^+ values were 4.28, 3.65, 3.40 and 3.25 mmol/l. Extraction was performed for 60 min at each concentration of the analytes in the subdermal phase; at the end of each hour, the current was stopped to allow the cathode solution to be removed for

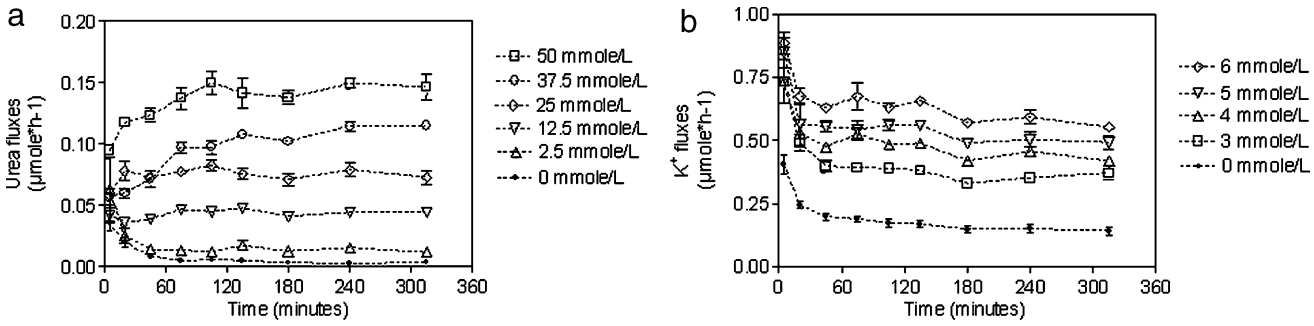

Fig. 1. **a** Reverse iontophoretic extraction fluxes of urea as a function of time and subdermal urea concentration. Each point represents the mean ± SD (n=3–11). **b** Reverse iontophoretic extraction fluxes of potassium as a function of time and subdermal potassium concentration. Each point represents the mean ± SD (n=4).

Table I. Regression of Reverse Iontophoretic Extraction Fluxes of Urea with the Corresponding Subdermal Concentrations, as a Function of Time of Current Passage

Time (min)	v (µl.h^{-1}) (a)	r^2 (a)	K' (l/µmol) (b)	r^2 (b)
10	0.6 ± 0.4[a]	0.10	0.16 ± 0.08	0.15
30	1.7 ± 0.2	0.73	0.36 ± 0.03	0.80
60	2.1 ± 0.1	0.86	0.42 ± 0.02	0.94
90	2.4 ± 0.1	0.91	0.48 ± 0.02	0.95
120	2.6 ± 0.1	0.93	0.49 ± 0.02	0.96
150	2.6 ± 0.1	0.91	0.50 ± 0.02	0.96
210	2.6 ± 0.2	0.89	0.54 ± 0.01	0.98
270	2.8 ± 0.1	0.97	0.57 ± 0.02	0.97
360	2.8 ± 0.1	0.96	0.71 ± 0.05	0.88

At each time point, the fluxes of urea, or the ratio of these fluxes to that of the "internal standard" (Na^+), at different subdermal concentrations, were fitted, respectively, (a) to the simple linear relationship in Eq 2, and (b) to the 'normalized' flux expression, Eq 4. The values of the proportionality constants (v and K', respectively), expressed as the mean ± SD from 3 to 11 separate experiments, together with the corresponding determination coefficients (r^2) are presented.
[a] Value of the constant is not significantly different from zero.

analysis and replaced with fresh electrolyte (2 ml of 10 mmol/l L-histidine) and the subdermal solution to be replaced with the next (lower) concentrations of the analytes.

Sample Analysis

A diacetylmonoxime colorimetric method, previously used for the determination of very low concentrations in seawater and other biological samples (31) was adapted for the analysis of urea in the reverse iontophoretically extracted solutions. The samples and appropriate 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. Immediately thereafter, 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) were added to each well. The plate was wrapped in aluminium foil and shaken for 10 min. The samples were then maintained at 85°C for 75 min, before cooling for 5 min in an ice-bath. Fifteen minutes later, the absorbance at 520 nm of the solution in each well was read (Spectra Mac 190, Molecular Devices, Sunnydale, CA). Full details of the validation of the method are presented elsewhere (32).

Potassium and sodium in the reverse iontophoretically extracted samples were determined by flame photometry (IL 243 LED Flame Photometer, Instrumentation Laboratory, Milan).

Table II. Regression of Reverse Iontophoretic Extraction Fluxes of K^+ with the Corresponding Subdermal Concentrations, as a Function of Time of Current Passage

Time (min)	γ (µl.h^{-1}) (a)	r^2 (a)	K' (l/µmol) (b)	r^2 (b)
10	57 ± 24	0.28	6.7 ± 3.2[a]	0.23
30	59 ± 21	0.36	8.7 ± 1.3	0.75
60	78 ± 7.7	0.88	10 ± 0.6	0.95
90	86 ± 16	0.69	12 ± 1.2	0.88
120	80 ± 6.7	0.91	10 ± 0.4	0.98
150	90 ± 6.4	0.93	10 ± 0.5	0.97
210	79 ± 5.5	0.94	10 ± 0.5	0.97
270	77 ± 9.2	0.83	11 ± 0.5	0.97
360	62 ± 8.6	0.79	8.7 ± 0.7	0.93

At each time point, the fluxes of potassium, or the ratio of these fluxes to that of the "internal standard" (Na^+), at different subdermal concentrations, were fitted, respectively, (a) to the simple linear relationship $J_{K^+}=\gamma.C_{K^+}$, and (b) to the 'normalized' flux expression, Eq 4. The values of the proportionality constants (γ and K', respectively), expressed as the mean ± SD from four separate experiments, together with the corresponding determination coefficients (r^2) are presented.
[a] Value of the constant is not significantly different from zero.

Data Analysis

Iontophoretic fluxes are reported as mean values ± standard deviation (SD). The slopes of the regression lines between extracted fluxes and subdermal concentrations are reported as mean values ± the 95% confidence interval (CI). Data manipulation, and linear and non-linear regressions, used Graph Pad Prism V 4.0 (Graph Pad Software, Inc., San Diego, CA); the significance of linear regressions was assessed by ANOVA at the level of $p<0.05$.

RESULTS AND DISCUSSION

The reverse iontophoretic extraction of urea, measured in the first series of experiments as a function of time and of the subdermal concentration of the analyte, is shown in Fig. 1a. The transport requires between 1 and 2 h to become stable. Noticeably, for the lower subdermal concentrations (and for the urea-free control), urea extraction is initially high before falling to lower, more constant rates, indicating the presence of a significant urea reservoir in the skin. This observation has been previously observed (26) and must, at least in part, be due to the known presence of urea in the skin's "natural moisturizing factor" (33,34). The fact that the initial extraction rates are rather independent of urea concentration makes the previously reported (10) correlation between the urea extracted by only 5 min of reverse iontophoresis and the blood level in human subjects difficult to understand. Elsewhere, and consistent with the *in vitro*

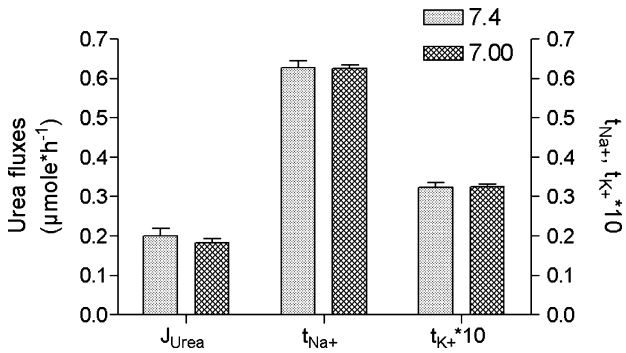

Fig. 2. Comparison of reverse iontophoretic extraction fluxes of urea, and the concomitant transport numbers (t) of Na^+ and K^+, determined at pH 7.4 and at pH 7.0.

results reported here, an urea skin 'reservoir' has been observed in healthy human volunteers (26).

The correlations between urea extraction flux and subdermal concentration evolved as a function of the experiment's duration (Table I). During the first 60 min, depletion of the urea 'reservoir' adversely impacted the quality of the anticipated dependence. Thereafter, the correlation was excellent with a slope, which corresponds to the electroosmotic flow (v) (Eq. 2), of about 2.7 µl/h. Using Na^+ as an internal standard led to better correlations between the ratio of urea to Na^+ fluxes and the subdermal urea concentration as described in Eq. 4. With this approach, a reasonable r^2 was achieved within the first hour of iontophoresis; the value of K' deduced was approximately 0.5 l/µmole. The extraction rates observed *in vivo* (26), when normalized with respect to the current applied, are of the same order (for comparable subdermal levels) as those reported in Fig. 1a (34.8±9.4 $\mu l.h^{-1}.mA^{-1}$ versus 28± 12 $\mu l.h^{-1}.mA^{-1}$ in this study); in contrast, the reported *in vivo* extraction rates from normal and renally-impaired subjects after only 5 min of iontophoresis were approximately 50–200 times higher (10), almost certainly reflecting depletion of the aforementioned skin 'reservoir.'

Reverse iontophoresis extraction of K^+ followed a pattern rather similar to that of urea (Fig. 1b) but the current had to be increased to 0.4 mA because results obtained with 0.2 mA were not conclusive (data not shown). At the initiation of the current passage, the flux was high and relatively insensitive to the subdermal K^+ concentration. Even when no exogenous K^+ was introduced in the iontophoresis cell, there was an easily measurable extraction of endogenous K^+, at first elevated and then quite constant, over the 6-h duration of the experiment. The correlation between the iontophoretix flux of K^+ and the subdermal concentration of the cation became linear after 90–120 min of extraction. Normalization via the Na^+ "internal standard" strategy reduced this time to ~ 1 h (Table II).

The results of the experiments performed to evaluate the effect of acidosis on the reverse iontophoretic extraction of urea and K^+ are summarized in Fig. 2. The transport of urea was not significantly altered by reducing the pH of the

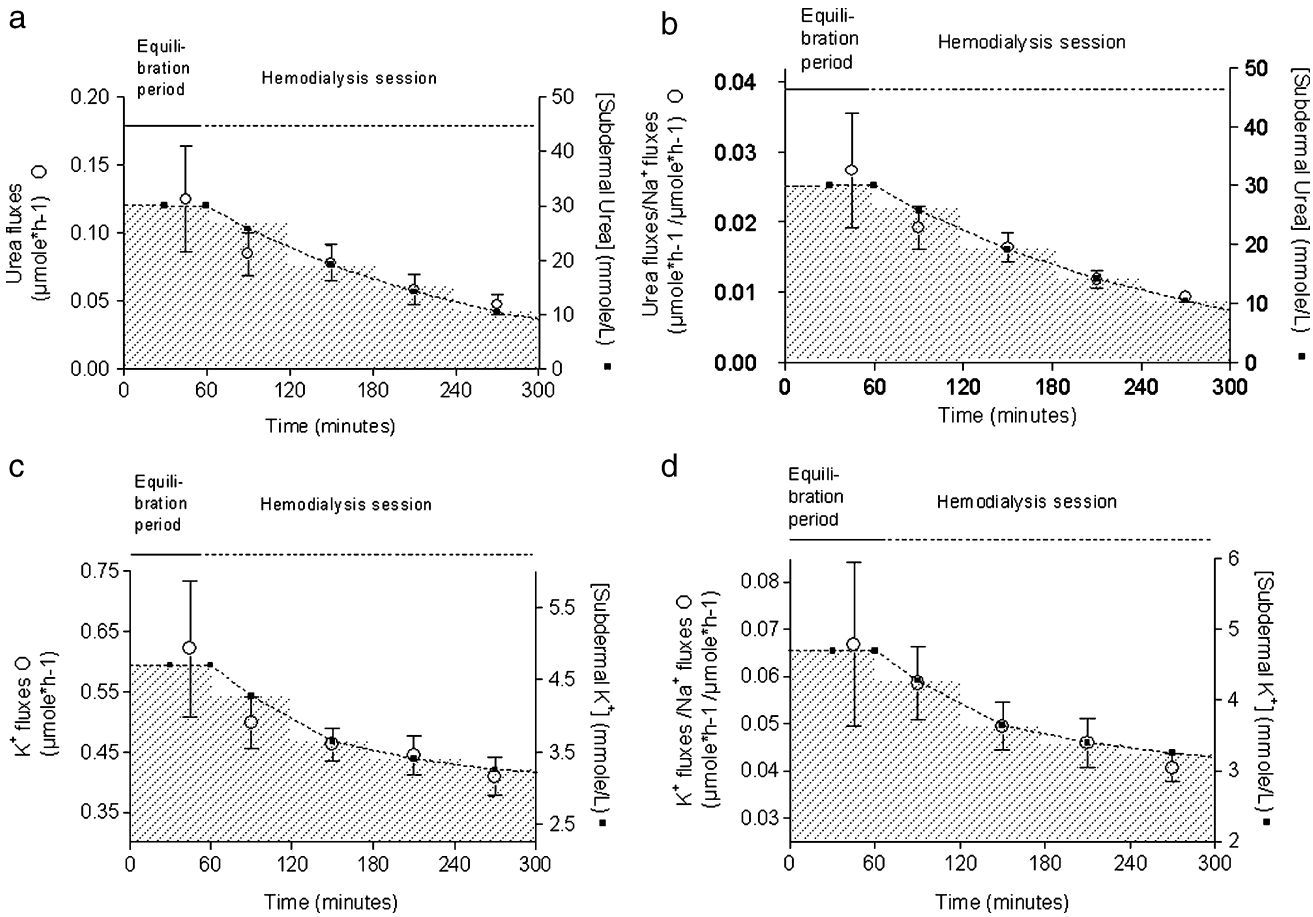

Fig. 3. **a** Reverse iontophoretic extraction fluxes of urea (*circle*), determined as a function of time as the subdermal concentration of the analyte (*filled square*) was progressively lowered over 6 h, to simulate a hemodialysis procedure. Each data point is the mean ± SD from four separate experiments. **b** Ratios of the reverse iontophoretic extraction flux of urea to that of Na^+ (*circle*), determined as a function of time as the subdermal concentration of urea (*filled square*) was progressively lowered over 6 h, to simulate a hemodialysis procedure. Each data point is the mean ± SD from four separate experiments. **c** Reverse iontophoretic extraction fluxes of K^+ (*circle*), determined as a function of time as the subdermal concentration of the analyte (*filled square*) was progressively lowered over 6 h, to simulate a hemodialysis procedure. Each data point is the mean ± SD from four separate experiments. **d** Ratios of the reverse iontophoretic extraction flux of K^+ to that of Na^+ (*circle*), determined as a function of time as the subdermal concentration of potassium (*filled square*) was progressively lowered over 6 h, to simulate a hemodialysis procedure. Each data point is the mean ± SD from four separate experiments.

subdermal compartment from 7.40 to 7.00. Equally, the transport number of K^+, and that of Na^+, were unchanged and consistent with data previously reported (7,9,25,35). It is unlikely, therefore, that the occurrence of acidosis *in vivo* could compromise the feasibility of using reverse iontophoresis as a tool to monitor patients with severe renal disease.

The data from the simulated "hemodialysis" experiments are collected in Fig. 3a and c, for urea and potassium, respectively. For both analytes, the reverse extraction fluxes faithfully tracked the progressive decrease in the subdermal concentration as a function of time, and the variation in the extraction fluxes decreased with increasing time of iontophoresis. The use of Na^+ as internal standard yielded no significant benefit (Fig. 3b and d). Previous work using microdialysis has shown that the urea concentration in the intersitital fluid closely follows that in the blood (18). Given that reverse iontophoresis is sampling the interstitial fluid in the skin, it seems reasonable to suggest that this technique may therefore offer a minimally invasive approach with which to track urea concentration in the blood *in vivo*.

CONCLUSION

Reverse iontophoresis was accurate to perform punctual measurements and to track decreasing urea and potassium subdermal concentrations *in vitro*. If, similar to the GlucoWatch®, a miniaturized system, including the determination of and display of analyte concentrations, could be developed for urea or potassium, the technique could be useful to screen patients at risk of renal disease and to follow up patients with renal failure as well as to monitor hemodialysis session non-invasively with immediate reading of the results. The wide range of applications of this technique, combined with the advantages of using alternative technique to venepuncture (decreased pain and risk of infection,...), highlights the potential of the technique. The ability to track in parallel, or independently, the levels of urea and potassium adds significantly to the clinical relevance of the research described and justifies the next, logical step to perform experiments *in vivo* in patients suffering from renal failure both to follow up urea and potassium levels and to monitor their status during hemodialysis.

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