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Iontophoretic transdermal sampling of iohexol as a non-invasive tool to assess glomerular filtration rate

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Abstract

Purpose: To explore the potential of non-invasive reverse iontophoresis transdermal extraction of iohexol as a marker of glomerular filtration rate.

Methods: A series of *in vitro* experiments were undertaken to establish the feasibility of iohexol reverse iontophoresis and to determine the optimal conditions for the approach. Subsequently, a pilot study in paediatric patients was performed to provide proof-of-concept.

Results: The iontophoretic extraction fluxes of iohexol *in vitro* were proportional to the marker subdermal concentration and the reverse iontophoretic technique was able to track changes dynamically in simulated pharmacokinetic profiles. Reverse iontophoresis sampling was well tolerated by the four paediatric participants. The deduced values of the iohexol terminal elimination rate constant from transdermal reverse iontophoresis sampling agreed with those estimated by conventional blood sampling.

Conclusions: Reverse iontophoretic transdermal extraction fluxes mirrored the subdermal concentration profiles of iohexol, a relatively large neutral marker of glomerular filtration both *in vitro* and *in vivo*. The efficiency of extraction *in vivo* was well predicted by the *in vitro* model used.

Keywords: iontophoresis, iohexol, GFR, non-invasive monitoring, transdermal sampling

1. Introduction

The assessment of kidney function is essential to several clinical procedures, including dose and fluid individualization, the assessment of nephrotoxicity, and the evaluation of chronic kidney disease (CKD) progression (1,2). Glomerular filtration rate (GFR), normally considered the best marker of kidney function, is assessed through different approaches (3). For example, estimated GFR is typically based on serum creatinine measurements and is widely used due to its simplicity and practical convenience (2). However, not all the non-GFR determinants of creatinine concentration are accounted for in this approach limiting its usefulness for some patient populations (2,4). On the other hand, measured GFR, based on the urinary or plasma clearance of exogenous filtration markers, is more informative but not routinely used due to the complexity of the procedure involved (2). Measured GFR is recommended, however, for staging CKD, for elderly and paediatric patients, for obese individuals and those with BMI < 18.5 Kg.m^{-2}, for those suffering from severe malnutrition, end-stage renal failure, cirrhosis, and in pregnant women. GFR is also measured when the kidney function is changing rapidly, or there is a nonstandard intake of creatinine or creatine (vegetarian diet and dietary supplements), and prior to the administration of renally eliminated nephrotoxic drugs, and before kidney donation (1-3, 5-7).

Inulin is considered the gold standard for GFR measurement but its practical use has several limitations. Therefore, alternative GFR markers have been proposed including iohexol (2,3,5,8), which is a non-radioactive, uncharged, and non-expensive X-ray contrast medium with low toxicity. Iohexol distributes to the extracellular space, is less than 2% plasma bound, and is excreted completely unmetabolized in urine exclusively by glomerular filtration (2,3,5,8). Iohexol has been described as the best alternative to inulin for precise,

accurate and relatively rapid determination of GFR and, being easily assayed by HPLC, has become the most commonly used contrast media for GFR measurements in Europe (1,4,6,8). Additionally, iohexol has been used for GFR measurements in paediatric (9,10), elderly (> 70 yr old) (11), and Type I and II diabetic (12) populations. In 2002, iohexol testing was considered (13) to be the standard method to assess GFR in Sweden, with 30 hospitals performing the procedure; the Lund University Hospital carried out 1500 iohexol determinations/year. At the Great Ormond Street Hospital in London, the number of iohexol procedures conducted per year ranges from 350 to 1000.

However, GFR measurement requires the intravenous administration (IV bolus) of iohexol followed by repeated blood sampling to characterize the clearance of the marker. This is an additional invasive procedure, therefore, to the numerous tests performed on certain paediatric patients (1,5,14). Venipuncture and IV cannula insertions are the most frequent causes of pain in hospitalized children (15), causing significant distress as well as increasing anxiety and fear with respect to future medical interventions (15,16). These procedures are often stressful for parents and carers too, and may be challenging to perform for the health care provider.

Reverse iontophoresis (RI) employs low intensity electrical current to enhance molecular transport across the skin, and has been used to non-invasively sample both drugs and clinical markers such as lithium and glucose across the skin (17-21). The potential of RI to diagnose and monitor CKD via the *in vivo* monitoring of urea has also been explored (22,23). RI discriminated between healthy and CKD patients and was able to track the decline in systemic urea during haemodialysis.

As stated above, the measurement of GFR requires the clearance of the selected marker to be determined once its systemic distribution has been completed (5,14). Such

pharmacokinetic profiling has been demonstrated for lithium (24), the apparent elimination rate of which was shown to be directly proportional to the RI extraction flux. While lithium is a small cationic drug, iohexol is a much larger (821.14 Da), neutral compound, the iontophoretic transport of which occurs by electroosmosis alone (18), the same mechanism that operates when RI is used for glucose monitoring (17,18,20,21). The challenge of the research described here is to demonstrate that RI is a valid non-invasive tool to exploit the use of iohexol as a marker of GFR. Following a series of *in vitro* experiments to establish the feasibility and optimal conditions of the procedure, a pilot study in paediatric patients was performed to provide the proof-of-concept.

2. Materials and methods

2.1 Chemicals

Iohexol (98% pure), acetaminophen (≥99%), silver wire (99.99%), and silver chloride (99.999%) were purchased from Sigma Aldrich (Gillingham, UK). Tris base (α, α, α-Tris-(hydroxymethyl)-aminomethane), sodium chloride, and potassium chloride were from Acros (Geel, Belgium). Hydrochloric acid (37 %w/w), methanol, acetonitrile (HPLC grade) and trifluoroacetic acid were obtained from Fisher Scientific (Loughborough, UK). All reagents were at least analytical grade and deionised water (resistivity ≥ 18.2 MΩ.cm, Barnsted Nanopure Diamond™, Dubuque, IA) was used for the preparation of all solutions.

2.2 *In vitro* experiments

2.2.1 Skin

Abdominal fresh pig skin was obtained from a local slaughterhouse and dermatomed

(Zimmer™ Electric Dermatome, Dover, Ohio; nominal thickness 750 µm). Skin sections were wrapped individually in Parafilm™ and kept frozen (-20°C) until use. Just before the experiment, the skin was thawed at room temperature for 30 minutes and visible hairs were carefully cut with scissors. The skin was then mounted directly onto the diffusion cells.

2.2.2 Iontophoresis

Iontophoretic experiments (n = 4-12) used Ag/AgCl electrodes connected to a power supply (KEPCO 1000M, Flushing, NY, USA), which delivered direct, constant current. Electrode and subdermal solutions were magnetically stirred.

Fixed-subdermal concentration extraction: The skin was sandwiched between the two compartments of side-by-side diffusion cells (0.71 cm^2, 3 ml) with the stratum corneum facing the cathode compartment. The skin was first hydrated for 30 minutes in the presence of a 7.4 pH, 25 mM Tris buffer in both compartments (no iohexol). The subdermal solution also contained 133 mM NaCl and 4 mM KCl.

After the 30-minute hydration period, the cathode compartment was emptied and refilled with fresh buffer. The anode solution was replaced with one of 5 iohexol concentrations ranging from 0.15 to 2.44 mM in the same buffer, reflecting the clinical range found in GFR measurements. A 0.36 mA current was applied for 6 hours. Every hour, the current was stopped; 1 ml from the cathode solution was withdrawn for analysis and replaced by fresh buffer solution. A passive diffusion control was also performed using the highest concentration of iohexol (2.44 mM).

Subsequently, the effect of a 3-hour iontophoretic pre-treatment of the skin on iohexol extraction was examined. For this, the skin was hydrated as before and then exposed to

0.36 mA for 3 hours. Acetaminophen (7.5 mM) was present in the anode solution during this pre-treatment allowing electroosmotic flow to be monitored. After the pre-treatment, the anode solution was replaced by Tris buffer (25 mM Tris, 133 mM NaCl, 4 mM KCl; pH 7.4) containing 2.44 mM iohexol and 7.5 mM acetaminophen. The current was then re-applied for a further 6 hours and hourly samples were taken. A passive diffusion control was performed in a separate experiment using skin, which had been exposed to a 3-hour constant current pre-treatment (0.36 mA) in the absence of iohexol.

<u>Pharmacokinetic simulations</u> used side-by-side three compartment diffusion cells (1.02 cm^2). Anode and cathode electrode chambers (1.9 ml) were each separated from a central compartment (3.75 ml) by a piece of skin oriented so that the stratum corneum faced the electrode chambers. The electrode chambers contained 25 mM Tris (pH 7.4) in the cathode, and 90 mM NaCl (necessary for electrochemical reactions) in 25 mM Tris buffer (pH 7.4) in the anode compartment. The subdermal compartment was continuously infused with Tris buffer (25 mM Tris, 133 mM NaCl, 4 mM KCl, pH 7.4) using a syringe pump (Genie 8, Kent Scientific Corporation, Torrington CT, USA) to allow the progressive clearance of iohexol from the subdermal compartment. Two sets of experiments were performed:

The skin was hydrated for 30 minutes (no iohexol) after which the solutions were refreshed and 0.31 mM iohexol was introduced into the subdermal compartment. At his point, the syringe pump started to infuse the subdermal compartment at 1.5 ml/h rate (i.e., simulation of a mono-exponential decay of iohexol) and a 0.5 mA constant current was applied for 6 hours. Every 30 minutes, the current was stopped and the entire cathodal solution was replaced with fresh buffer. Samples (10 μl) were also collected from the subdermal compartment at the mid-point of each iontophoretic sampling period.

The second set of experiments pre-exposed the skin to 3 hours of constant current (0.5 mA) in the absence of iohexol. After the pre-treatment period, all solutions were refreshed and iohexol (at 1.22 or 6.04 mM) was introduced into the subdermal compartment. Constant, direct current (0.5 mA) was applied for 6 hours. One experiment simulated another mono-exponential profile decay from an initial 1.22 mM subdermal concentration using a syringe pump flow of 1.0 $ml.h^{-1}$. The final experiment simulated a two-compartment IV bolus decay from an initial subdermal concentration of 6.04 mM and the pump flow rate was modified from 3.0 $ml.h^{-1}$ in the first three hours to 1.0 $ml.h^{-1}$ for the subsequent three hours. In all cases, samples were collected from both the subdermal and cathodal compartment as before.

2.2.3 Sample analysis

Iohexol and acetaminophen were quantified simultaneously by HPLC with UV detection (254 nm). The method was modified from a previous publication [25] and used an HPLC system (Dionex, Sunnyvale, CA) comprising a P680 pump with ASI-100 autosampler, TCC-100 thermostated column compartment, PDA-100 diode array detector, and an Acclaim 120, C18 (150 x 4.6 mm, 5 μm) reversed-phase column (Dionex, UK) thermostated at 60°C. The mobile phase, 13.5 mM trifluoroacetic acid (pH 2.2): methanol (95:5 v/v), was pumped through the system at 1 ml/min.

2.2.4 Data analysis and statistics

Data analysis and regressions were performed using Graph Pad Prism V.5.00 (Graph Pad Software Inc., CA, USA). Unless otherwise stated, data are represented as the mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$.

Extraction fluxes (J_{IOX}) were calculated as the amounts transported during an extraction period divided by the duration of the sampling interval. The apparent convective solvent flow or extraction efficiency (κ_{IOX}) for a given subdermal concentration of iohexol (C_{IOX}) and extraction flux (J_{IOX}) was estimated as:

$$J_{IOX} = \kappa_{IOX} \times C_{IOX} \qquad \text{Eq.1}$$

The corresponding, equivalent, calculation and equation were used to estimate J_{ACM} and κ_{ACM}. The slopes of linear regressions of Eq. 1 corresponding to different iontophoresis times during the fixed concentration experiments were compared by an ANCOVA test. Comparison of fluxes at different times used repeated-measures ANOVAs followed by a Tukey's post-test. Two-way repeated measures ANOVAs followed by Bonferroni post-test were used to evaluate the effect of skin pre-treatment on extraction fluxes and to compare the iontophoretic extraction efficiencies of iohexol and acetaminophen. When relevant, the values of κ_{IOX} are reported normalized by the current intensity to facilitate comparison among different experimental conditions.

In the case of experiments simulating a mono-exponential decay, the linear sections of the semi-logarithmic representation of the subdermal concentrations (C_{IOX}) versus time and of the extraction fluxes (J_{IOX}) versus time were fitted to the equations:

$$lnC_{IOX} = lnC_{0,IOX} - K_e \times t \qquad \text{Eq.2}$$

and

$$lnJ_{IOX} = ln(\kappa_{IOX} \times C_{0,IOX}) - K_e \times t \qquad \text{Eq.3}$$

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K_e, $C_{0,IOX}$ and ($\kappa_{IOX}.C_{0,IOX}$) were obtained by fitting the experimental data to Eqs. 2 and 3. κ_{IOX} was estimated from C_{IOX} and J_{IOX}. The *in vitro* "volume of distribution" (V_d) and clearance (Cl) were determined using:

$$C_{0,IOX} = D/V_d \qquad\qquad Eq.4$$

and

$$K_e = Cl/V_d \qquad\qquad Eq.5$$

where D is the amount of iohexol introduced into the subdermal compartment of volume (V_d) at t = 0. An additional, model independent, estimation of clearance was made using:

$$Cl = D/AUC_0^{\infty} \qquad\qquad Eq.6$$

AUC_0^t was calculated by the trapezoidal rule and AUC_t^{∞} was determined as the ratio between the last measured concentration and the rate constant (K_e).

When the experiment involved a change in the pump flow rate the subdermal IOX concentration was described by:

$$C_{IOX} = Z_{i=1,2} \times exp(-K_{i=1,2} \times t) \qquad\qquad Eq. 7$$

where Z_1 and K_1 apply at t = 0 - 3 hours, and Z_2 and K_2 apply at t = 3 - 6 hours. The semi-logarithmic representation of the data from these experiments exhibits two linear sections, which were fitted independently to find the values of Z_1, Z_2, K_1 and K_2.

Statistical comparison of the kinetic parameters obtained from the subdermal concentration and iontophoretic extraction data were performed with two-tailed Mann-Whitney tests.

2.3 In vivo pilot study

2.3.1 Subjects

Four children (9.1, 13, 7.4, and 12 years old; 1 female and 3 males) undergoing a routine iohexol GFR test at Great Ormond Street Hospital for Children (GOSH) participated in the study. Approval was granted from the Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee, and prior written consent and assent from the parent and child, respectively, were obtained before beginning the study. Participants did not have any skin condition, such as eczema, irritated or damaged skin.

2.3.2 *In* vivo experimental protocol

The routine GFR test involved the bolus intravenous administration of 2 ml of Omnipaque 300 (Nycomed Amersham plc, Bucks., UK), equivalent to 1294 mg iohexol. Subsequently, two 1 mL blood samples were taken from the subject at approximately 3 and 4 hours post-injection (Figure 1.b). The samples were analysed for iohexol by the GOSH clinical chemistry laboratory.

Reverse iontophoresis was carried out in parallel on the non-dominant arm of the participant. The arm was first cleaned with an alcohol wipe and then allowed to dry. Constant, direct current (Figure 1.a) was delivered from a "Phoresor II auto" device (PM850, Iomed, Salt Lake City, Utah) to the electrode patches (Iogel small, Iomed) affixed to the subject's skin. The anode was a dispersive pad (22.6 cm^2) incorporating a silver/silver chloride electrode in karaya gel. The cathode (7.2 cm^2) was a silver chloride electrode integrated into a dried gel, which was hydrated with 1.3 ml of ultrapure water. The Phoresor delivered 0.5 mA (0.07 mA/cm^2 at the cathode sampling site) for 5 hours except for participant 3 for whom the applied current was 0.3 mA, beginning 0.5 hours after the

intravenous dose of iohexol. Both patches were refreshed after 2 and 4 hours of current passage, and the cathodal patch was additionally replaced at 3 hours (Figure 1.b). When the patches were exchanged, the current was interrupted briefly.

Participants completed a short questionnaire just after the start of iontophoresis and towards the end of the experiment. The questionnaire used the Wong-Baker faces pain scale [26] to indicate the level of sensation experienced during iontophoresis. Subjects were also asked whether transdermal iontophoresis was preferred over blood sampling.
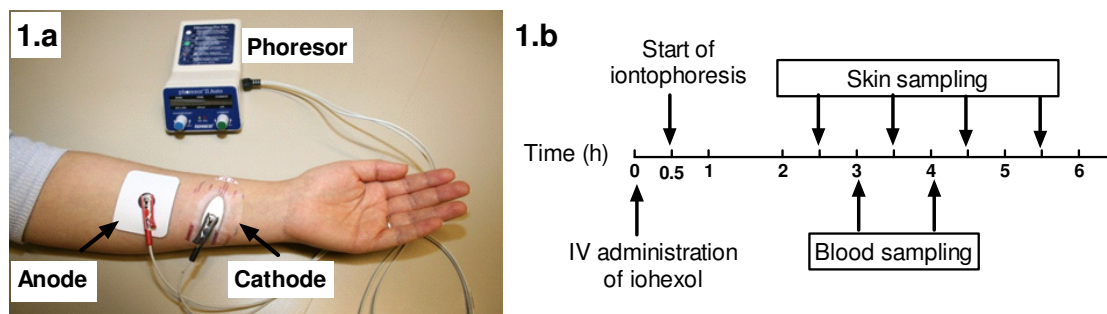
Figure 1

Figure 1: Left panel: Experimental set-up used in the in vivo RI pilot study. Iohexol was extracted at the cathodal patch. Right panel: Timeline for the *in vivo* study starting with IV administration, the times at which iontophoresis was started and transdermal and blood samples were taken are also indicated.

2.3.3 *In vivo* sample analysis

The cathodal patches were immersed in 7 ml of water and shaken at 240 rpm (HS260 Basic, IKA, Sigma Aldrich, Gillingham, UK) for 24 hours. The extracts were filtered by centrifugation

(45 minutes at 7500 RCF) using Amicon ultrafiltration tubes (MWCO 5 kDa, Millipore, Watford, UK).

Iohexol was quantified by HPLC. The method used a Shimadzu system (LC-2010A HT, Kyoto, Japan) comprising an autosampler, a UV diode array detector set at 254 nm, and a HiQ-SilTM C18 (250 x 4.6 mm, 5 µm) reversed-phase column (Jasco, UK) thermostated at 40°C. The mobile phase, water:acetonitrile (70:30, %v/v), was pumped at 1 ml/min flow rate. The effective run for each injected sample (20 µl) was 7 minutes and a wash step (water:acetonitrile, 20:80, %v/v) for 38 minutes followed by a 10 minute column re-equilibration with mobile phase.

Sodium and potassium in the extracts were analysed by ion chromatography with suppressed conductivity detection. The method, based on previous work [27], used an IC system (Dionex, Sunnyvale, CA) comprising a GP-50 gradient pump, an AS-50 autosampler and thermal compartment, and an ED-50 electrochemical detector. The mobile phase, 20 mM methanesulfonic acid, was pumped at 1 ml/min through a Dionex IonPacTM CS12A (250 x 4 mm) column thermostated at 30°C and connected to a Dionex CSRS Ultra II suppressor (4 mm) set at a current of 80 mA.

2.3.4 Calculations and statistical analysis of *in vivo* data

Data analysis and regression were performed using Graph Pad Prism V.5.00 (Graph Pad Software Inc., CA, USA). The slope and intercept of regression lines are expressed as the best-fit value ± standard error of the regression (SE). The extraction fluxes of sodium and potassium at different times of iontophoresis were compared using repeated-measures ANOVA test. The level of significance was set at $p < 0.05$. The blood concentrations of

iohexol were plotted at the exact times of blood sampling; whereas the transdermal extraction fluxes were plotted at the midpoint of the respective extraction interval. As the sampling times of blood measurements did not coincide with the sampling times of reverse iontophoresis, the corresponding plasma concentrations, when correlated to iontophoretic extraction fluxes, were adjusted by interpolation (i.e., using the individual value of β determined through blood sampling).

Sodium and potassium transport numbers were calculated using Faraday's law :

$$J_j = [I \times t_j]/\{F \times z_j\} \qquad\qquad \text{Eq. 8}$$

where I is the intensity of current applied, F is Faraday's constant, and J_j, t_j and z_j are the iontophoretic flux, transport number and valence, respectively, of the ion "j" (17-19).

The terminal rate constant (β) was estimated from the iohexol concentrations ($C_{p,IOX,\,t1}$ and $C_{p,IOX,\,t2}$) in the blood samples withdrawn at approximately 3 (t_1) and 4 (t_2) hours post-injection using the relationship:

$$Ln(C_{p,IOX,\,t1} / C_{p,IOX,\,t2}) = \beta \times (t_2 - t_1) \qquad\qquad \text{Eq. 9}$$

The extraction fluxes of iohexol (J_{IOX}) were anticipated to follow the changes in the marker's blood levels (i.e., $J_{IOX} = \kappa \times C_{p,IOX}$) once iohexol had reached the equilibrium of distribution across the extracellular fluid (~2-3 hours post-injection). Hence,

$$Ln(J_{IOX,t1} / J_{IOX,t2}) = \beta \times (t_2 - t_1) \qquad\qquad \text{Eq. 10}$$

β was also estimated after calibration of iohexol's iontophoretic extraction flux (J_{IOX}) with that of a potential internal standard candidate (J_{IS}, either sodium or potassium), simultaneously extracted with iohexol. In other words, $\{J_{IOX}/J_{IS}\} = \kappa' \times C_{p,IOX}$, and therefore:

$$ln\{(J_{IOX, t1}/J_{IS, t1}) /(J_{IOX, t2}/J_{IS,t2})\} = \beta \times (t_2 - t_1) \quad \text{Eq. 11}$$

where J_{IS} is expected to be constant given the constant systemic concentration of the internal standard (18, 19, 28). Finally, the *in vivo* efficiencies of iohexol extraction, κ and κ' were estimated.

3. Results

3.1 *In vitro* experiments

Fixed subdermal concentration extraction: Passive extraction fluxes of iohexol (0.06 ± 0.04 nmol/h over 6 hours for a 2.44 mM subdermal concentration) were negligible compared to the nearly 80 times higher iontophoretic fluxes. Moreover, even when a smaller subdermal concentration (0.15 mM, i.e., one-sixteenth of that used in the passive diffusion experiment) was iontophoresed, an extraction flux of 0.31 ± 0.08 nmol/h was observed. Clearly, therefore, iontophoresis offers a much more efficient sampling technique than passive diffusion.

Iohexol fluxes in fixed subdermal concentration experiments increased with time for each concentration tested (Figure 2, left panel) and appeared to be reaching a plateau after 6 hours. The right panel of Figure 2 shows the linear correlation found between iohexol subdermal concentration and iontophoretic extraction flux; the proportionality improves with time of iontophoresis (e.g., r^2 = 0.88 at 6 h). In other words, the extraction efficiency ($κ_{IOX}$ $\mu l.h^{-1}$) stabilizes with time (Table I). No significant differences (ANCOVA test) between the slopes from the fourth hour of iontophoresis were found after which the pooled average value of $κ_{IOX}$ was 1.81 ± 0.13 $\mu l.h^{-1}$ (mean ± SE) or 5.03 $\mu l.h^{-1}.mA^{-1}$.

Figure 3 shows the effect of a 3 h - 0.36 mA iontophoresis pre-treatment on iohexol extraction and that of ACM, which was used as a marker of electroosmotic flow. The pre-treatment slightly increased the total extraction flux of iohexol and values measured at 2 through 5 hours were significantly higher than without pre-treatment ($p < 0.001$ (2 h) and $p < 0.05$ (5 h)) (Table I). However, there was no statistical difference at 6 hours. A small increase ($p < 0.001$) in the passive diffusion, from $J_{6h} = 0.06 \pm 0.04$ to 0.24 ± 0.04 nmol/h was found when the skin was pre-iontophoresed but the resulting contribution remained negligible compared to the electrotransport. Overall, the pre-treatment shortened the time for iohexol fluxes to plateau by about 1 hour. The extraction efficiencies for IOX and ACM differed significantly (Table I). For example, $\kappa_{IOX,6h} = 1.96 \pm 0.51$ $\mu l.h^{-1}$ (5.4 ± 1.4 $\mu l.h^{-1}.mA^{-1}$) and $\kappa_{ACM,3h} = 3.21 \pm 0.74$ $\mu l.h^{-1}$ (8.9 ± 2.0 $\mu l.h^{-1}.mA^{-1}$). It should be noted that $\kappa_{IOX,6h}$ and $\kappa_{ACM,3h}$ correspond to 6 hours of skin current exposure and extraction time for both analytes).

"Pharmacokinetic profile simulation" extraction experiments: Three distinct experiments were performed. In the first, IOX was gradually cleared, using a constant syringe pump flow-rate, from the subdermal compartment in simulation of a first-order elimination process (Figure 4A). The second was an identical study except that the skin was subjected to a 3-hour iontophoretic pre-treatment (0.5 mA), prior to the initiation of IOX clearance (Figure 4B). In the third experiment (for which the skin was again pre-treated iontophoretically as before), IOX was eliminated in a biphasic manner by altering the perfusion of the subdermal compartment after 3 hours (Figure 4C). The simulated kinetic parameters for each experiment were estimated by sampling the subdermal concentration (C_{IOX}) as a function of time and then compared to those deduced from the iontophoretic

extraction fluxes (J_{IOX}) (Table II). In all cases, there was a remarkably good agreement between the two determinations.

Extrapolation of the linear data of the plots in Figures 4A and 4B to t = 0 yields intercepts equal to the initial subdermal concentrations ($C_{0,IOX}$) of the analyte: 0.29 (±0.02) mM and 1.23 (±0.05) mM for no pre-treatment and iontophoresis pre-treatment, respectively, which are very close to the initial concentrations of the marker in the subdermal compartment (see Materials and Methods). Similarly, the values estimated for the volume of distribution and the clearance are comparable to the volume of the subdermal compartment and to the flow rate provided by the syringe pump.

3.2. *In vivo* pilot study

This pilot study examined the potential use of RI as a non-invasive sampling tool for IOX. For this, RI sampling was compared to the reference blood sampling method currently adopted in clinical practice. IOX was successfully extracted by transdermal RI. The blood concentrations of the marker and its iontophoretic extraction fluxes are presented semi-logarithmically in Figure 5 as a function of time post-injection. All extraction fluxes correspond to a current intensity of 0.5 mA except in the case of subject 3 for whom the applied current was lowered to 0.3 mA from the 5th minute post-current initiation.

The cathodal extraction fluxes of sodium and potassium ions were also measured at each iontophoretic sampling interval. Sodium extraction rates in the first interval (361 ± 22 $\mu g.h^{-1}.mA^{-1}$) were significantly lower than the subsequent measurements (544 ± 123 $\mu g.h^{-1}.mA^{-1}$), while potassium fluxes showed no statistical difference at any of the sampling intervals (mean value: 206 ± 38 $\mu g.h^{-1}.mA^{-1}$).

The terminal rate constant of IOX (β) for each subject was calculated from both blood and skin sampling data (Table III). Agreement between the values deduced was generally good: for three of the four subjects, β determined by blood sampling and RI differed by no more than a factor of 1.4; for the other volunteer (subject 1), the difference was 2.3-fold. The direct correlation between the iontophoretic extraction fluxes of iohexol and the corresponding blood concentrations (i.e., $J_{IOX} = \kappa_{IOX} \cdot C_{p,IOX}$) was reasonable (Figure 6A; $r^2 = 0.69$) with an average ($\pm$SD) κ_{IOX} value of 11.6 ($\pm$3.2) $\mu l.h^{-1}.mA^{-1}$. Individual κ_{IOX} values varied over the range 7.5-38.3 $\mu l.h^{-1}.mA^{-1}$, with the results for subject 1 being noticeably smaller than those for the other volunteers (Table III). The correlation improved when the internal standard concept (with Na^+) was used (i.e., $\{J_{IOX}/J_{IS}\} = \kappa_{Na+}' \cdot C_{p,IOX}$; see Figure 6B; $r^2 = 0.89$), and the mean ($\pm$SD) value of κ'_{Na+} was 27.4 ($\pm$4.0) $\mu l.mg^{-1}$ (Table III). Performing the internal standard calibration with K^+ resulted in a poorer correlation ($r^2 = 0.49$) with $\kappa'_{K+} = 42.1$ ($\pm$17.6) $\mu l.mg^{-1}$.

Blood sampling and iontophoresis were well tolerated and all four participants completed the trial. Subject 3 expressed discomfort with the original current setting (0.5 mA) and the intensity was therefore lowered to 0.3 mA from the 5th minute post-current initiation. Mild erythema at the patch application skin sites was noticed in all subjects and was more pronounced at the cathodal site. The adhesive material of the cathodal patch caused mild irritation to the skin of subject 2. Sensation to iontophoresis was evaluated by the universal Wong-Baker pain scale questionnaire (0 represents no pain; 5 reflects considerable discomfort). Current passage provoked the most intense feeling at the beginning of the experiment (pain levels were between 0 and 2); however, the sensation diminished substantially by the end of the study (pain levels falling to 0 or 1). Discomfort was principally attributed to patch removal and the tingling or itching sensation caused by current passage;

3 of the 4 subjects reported more tingling/itching below the larger, anode electrode pad. All participants expressed a preference for reverse iontophoresis over blood sampling.

4. Discussion

4.1 *In vitro* experiments

Iohexol is a hydrophilic (log P = -3.05), polar, neutral compound. The mechanisms contributing to its extraction flux during transdermal iontophoresis are passive diffusion and electroosmosis. Therefore, iontophoretic extraction towards the cathode was selected as the most efficient method for iohexol sampling. This is because, at physiological pH, the net charge on the skin is negative and electroosmosis proceeds in the direction of anode-to-cathode [29].

The first objective was to demonstrate a linear correlation between iohexol subdermal concentrations representing the clinical range typically observed in patients and the iontophoretic extraction fluxes. Table I and Figure 2 reveal that this was indeed the case and that the proportionality improved with time of iontophoresis (r^2 = 0.88 at 6 h). These findings are in line with previous studies concerning the RI extraction of other compounds [18,24,31,32]. The slopes of this linear relationship (κ_{IOX}, µl/h, Table 1) increased with time up to 4 hours after which the pooled, average (±SE) was 1.81 (±0.13) µl/h.

The values of κ_{IOX} and κ_{ACM} at different time points describe the iontophoretic extraction efficiency for IOX and ACM (Table 1) and, once stable, are primarily a reflection of the magnitude of the electroosmotic flow in the anode-to-cathode direction in the experimental conditions tested. The efficiency of extraction was higher for ACM; for example, after 6 hours of current application, $\kappa_{IOX,6h}$ was 1.96 ± 0.51 µl.h^{-1} (5.4 ± 1.4 µl.h^{-1}.mA^{-1}) and $\kappa_{ACM,3h}$ equalled 3.21 ± 0.74 µl.h^{-1} (8.9 ± 2.0 µl.h^{-1}.mA^{-1}) (Table I, Figure 3). However, these

differences are less than 2.5 fold despite the much larger molecular weight of IOX (821.14 Da) compared to ACM (151.17 Da) and correlate quite well with the ratio of the Stokes radii of the two compounds (1.1 nm (33) and 0.36 (34), for iohexol and acetaminophen, respectively. The absolute values of the transport efficiencies measured are also quantitatively similar to those determined in previous studies (35, 36) examining a series of uncharged polyethylene glycols spanning a wide range of molecular weight.
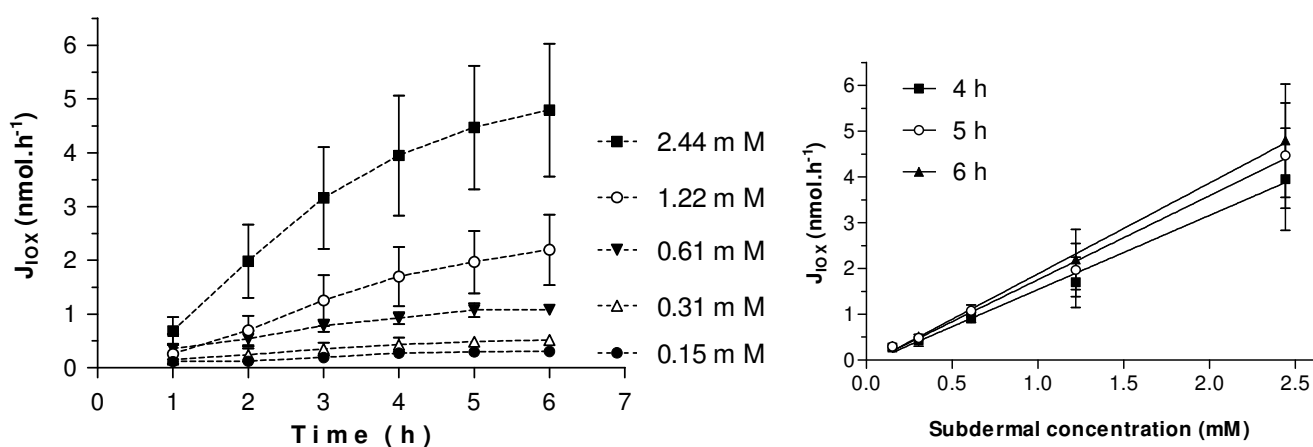
Figure 2

Figure 2: Left panel: RI extraction fluxes of iohexol as a function of time and subdermal IOX concentration. Right panel: proportionality observed between RI fluxes and IOX subdermal concentration after 4 hours. The data are represented as the mean ± SD (n = 4-6).

A second objective of these experiments was to determine the time required to reach stable extraction efficiency and whether this time could be shortened with a 3-hour, 0.36-mA iontophoresis pre-treatment. The practical implication of this observation is clear in that unless the value of κ_{IOX} is stable, the correspondence between J_{IOX} and C_{IOX}, and, hence, the

glomerular filtration rate is complicated. The 3 h pre-treatment was chosen because: (a) ACM fluxes tend to stabilise after ~3 hours of iontophoresis (Figure 3, Table I), and (b) when iohexol is used as a renal marker, its systemic distribution phase requires takes 2-3 hours (5, 14). Data from this distribution phase are not used in the calculation of GFR, and therefore the 2 to 3 hour period, from the moment of IOX administration, may profitably be used to allow transdermal extraction fluxes to stabilise (5, 14). However, while the pre-treatment slightly increased and accelerated the extraction flux of iohexol (Fig.3, Table I), 3 hours of iontophoresis were still required to reach steady extraction kinetics. The distinction between this observation and the behaviour of acetaminophen may reflect that additional factors, such as a differential accessibility of the two compounds to all iontophoretic transport pathways, contribute to the time necessary to reach steady-state extraction.
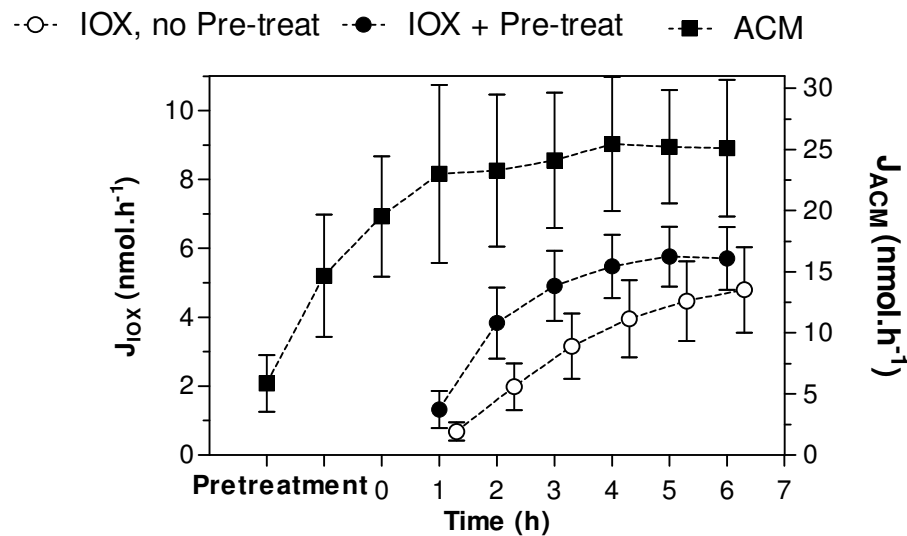
Figure 3

Figure 3: Effect of a 3-hour iontophoresis pre-treatment (0.36 mA) on iohexol (IOX) extraction and comparison with acetaminophen (ACM) fluxes. The subdermal concentrations of IOX and ACM were 2.44 mM and 7.5 mM, respectively. The symbols

representing "IOX without pre-treatment" are slightly nudged to the right for clarity. Data are presented as mean ± SD of n= 6-12.

The next series of experiments aimed to investigate iohexol RI extraction under dynamic conditions as the marker was progressively cleared from the subdermal compartment. First, a mono-exponential elimination process was simulated, with and without the 3-hour, 0.5-mA pre-treatment prior to the bolus administration of iohexol into the subdermal compartment. The resulting subdermal concentration profiles are in Fig.4, panels A-B, and show that, once the iohexol extraction stabilized (i.e., κ_{iox} is constant), the extraction flux data mirrored the corresponding subdermal concentration profile (in accord with Eqs 3 and 2, respectively) and the deduced terminal rate constants (K_e) were indistinguishable (Table II). Notably, for the experiments without and with iontophoretic pre-treatment, the deduced values of κ_{iox} were 4.8 (±0.5) $\mu l.h^{-1}$ (9.6 (±1.0) $\mu l.h^{-1}.mA^{-1}$) and 5.3 (±0.4) $\mu l.h^{-1}$ (10.6 (±2.0) $\mu l.h^{-1}.mA^{-1}$), respectively, and not significantly different from one another. In these dynamic conditions, the pre-treatment clearly shortened the time required for κ to stabilise (Fig.4, panels A and B).

In addition, RI sampling allowed good estimation of the other, simulated *in vitro* kinetic parameters (Table II). This ability has been demonstrated previously in a similar study that considered the use of RI for lithium monitoring (24). In contrast to iohexol, however, lithium is a small cation extracted almost exclusively, and much more efficiently, by electrorepulsion, (κ_{Li+} = 43 ± 6 $\mu L.h^{-1}$) with a much shorter time required to achieve a steady flux (24).

Because iohexol disposition *in vivo* is far better described by a two-compartmental model (3, 6, 14), the final set of *in vitro* experiments investigated whether a biphasic clearance of the marker from the subdermal compartment (as described by Eq. 7) could be tracked by RI and reflected in the measured extraction fluxes. As shown in Figure 4, panel C, reverse iontophoresis successfully and rapidly tracked the kinetic profile when the subdermal clearance was subjected to a step-change, and the deduced kinetic parameters (i.e., $Z_{i=1,2}$ and $K_{i=1,2}$, Table II) from the subdermal concentration and RI data were remarkably similar.

Taken together, the *in vitro* experiments convincingly demonstrated the ability of RI to track changes in the subdermal concentration of iohexol and to estimate relevant pharmacokinetic parameters of the marker, providing justification, therefore, for the subsequent, *in vivo* pilot study.

4.2 *In vivo* pilot study

This study compared, in 4 children, iohexol sampling by RI to the reference blood sampling method currently adopted in clinical practice. Iohexol was indeed successfully extracted by transdermal iontophoresis (Fig. 5) thereby allowing a preliminary assessment of the non-invasive approach. Sodium and potassium ions, present in the body at relatively constant concentrations and extracted simultaneously with iohexol at the cathode, were evaluated as possible internal standards with which to calibrate the extraction flux of the marker.

In agreement with earlier work (19, 37), J_{Na+} in the first sampling interval (15.7 ($\pm$1.0) $\mu mol.h^{-1}.mA^{-1}$) was significantly lower than the rather constant, subsequent measurements (23.6 ($\pm$7.6) $\mu mol.h^{-1}.mA^{-1}$) indicating an increase in transport number of the ion (or fraction of charge that it transports) from 0.42 ($\pm$0.03) to 0.63 ($\pm$0.14). This has been explained by depletion of the other ions present in the skin (38). The iontophoretic potassium flux was

effectively constant and similar at all sampling intervals with a mean value of 5.3 (±1.0) μmol.h^{-1}.mA^{-1} corresponding to a transport number 0.14 (±0.03). Compared to previous *in vivo* results (19, 37), however, the ion fluxes showed a greater variability (coefficients of variation of 23% and 19% for sodium and potassium, respectively) amongst the young subjects of the present study. Why this occurred is unclear given that the systemic levels of these cations are effectively constant. One possibility is that the semi-solid polymeric gel used as the cathodal sampling medium was difficult to remove completely at the end of the sampling interval, potentially leading to uncertainty that 100% recovery had been achieved. Self-evidently, further development of RI for iohexol monitoring will require improved formulation of the collection medium to ensure better consistency and reproducible recovery.

Figure 5 shows that, after 2-3 hours of current application, iohexol extraction fluxes decreased with time in accord with Eq. 10, and that the overlap with the decay in the blood concentrations of the marker (Eq. 9) was reasonably good. Although the number of *in vivo* data points is limited, their consistency with the *in vitro* results discussed above (Figure 4) is noteworthy. Reassuringly, and perhaps somewhat fortuitously, the 2-3 hours required for the systemic distribution of iohexol *in vivo* (3,14) appear to overlap quite closely with the same period needed for electroosmotic flow to stabilise after the initiation of iontophoresis. Subsequently, the terminal, elimination phase of the marker from the blood is well-tracked by its RI extraction flux from the subdermal interstitial fluid (and characterised by the same rate constant "β" (Eqs. 10 and 11)) (14). The values of the terminal rate constant (β) calculated in this way are in Table III. The RI results are, of course, based on the last three sampling intervals, which overlap temporally with the blood samples obtained.
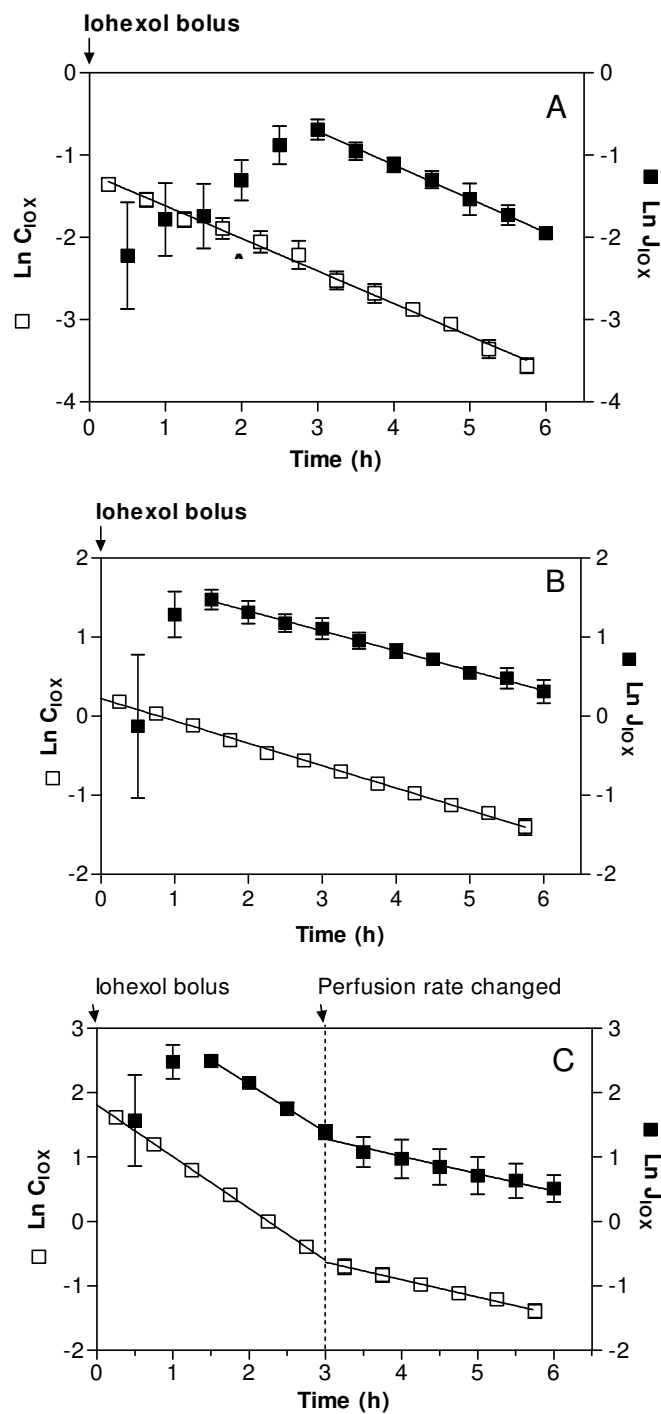
Figure 4

Figure 4: Profiles of lnC_{IOX} (open squares) and lnJ_{IOX} (filled squares) as a function of time corresponding to the "pharmacokinetic profile simulation" extraction experiments: C_{IOX} one exponential decline simulation without pre-treatment (panel A) and following a 3 hour iontophoresis pre-treatment (panel B) and C_{IOX} two-phase decline simulation with pre-treatment (panel C). The units for J_{IOX} and C_{IOX} were $nmol.h^{-1}$ and mM, respectively. Data are represented as the mean ± SD of n=4-5.

For 3 of the 4 subjects, the ratio of the derived values of β from the two approaches (blood sampling relative to RI) was less than 1.4; for the fourth patient, the ratio was 2.3 (Table III).

Because of the numerical method involved in estimating GFR from β (2,5) the ratio of the derived values for GFR estimated with blood and skin data mirror closely the ratios shown in Table III. The value of β determined by blood sampling is based on two measurements only and, self-evidently, any error in the evaluation of these samples will be manifest in the calculated β. Two advantages of the less invasive RI approach, therefore, are that the estimate of clearance can be based on multiple measurements rather than just two and that additional sampling times are easier to implement for patients with poor renal function. Table III also shows the values of β determined when the iontophoretic extraction fluxes of iohexol were normalized using those of two potential internal standards (Na^+ and K^+). While it appears that this strategy did not improve the overall precision of the estimates, the ratios of the derived values of β were consistently closer to unity. In general, β estimated by skin sampling was lower than that calculated from blood sampling. While the small number of subjects involved in this study precludes a definitive explanation for this observation, it is worth noting that RI samples the subdermal interstitial fluid rather than the blood and that the equilibration of iohexol concentrations within these two compartments may not be instantaneous (6,14,39).

The efficiency of iohexol extraction *in vivo* was evaluated for each of the four subjects based upon the two measured blood concentrations and the RI extraction fluxes either without (κ_{IOX}) or with (κ'_{Na+} and κ'_{K+}) the use of one of the two putative internal standards (Table III); average values of κ_{IOX} and κ'_{Na+} and κ'_{K} (for both Na^+ and K^+) were obtained via linear regression of these data (Figure 6). The correlation (r^2 = 0.69) between J_{IOX} and its

concentrations in the blood was reasonable yielding a mean value of κ_{IOX} of 11.6 (±3.2) $\mu l.h^{-1}.mA^{-1}$. The positive y-intercept of the regression supports the contention above that iohexol RI extraction samples the subdermal interstitial fluid, not the blood. Notably, the *in vivo* and *in vitro* (measured under dynamic conditions) values of κ_{IOX} were in close agreement indicative of the relevance and value of the *in vitro* model used.
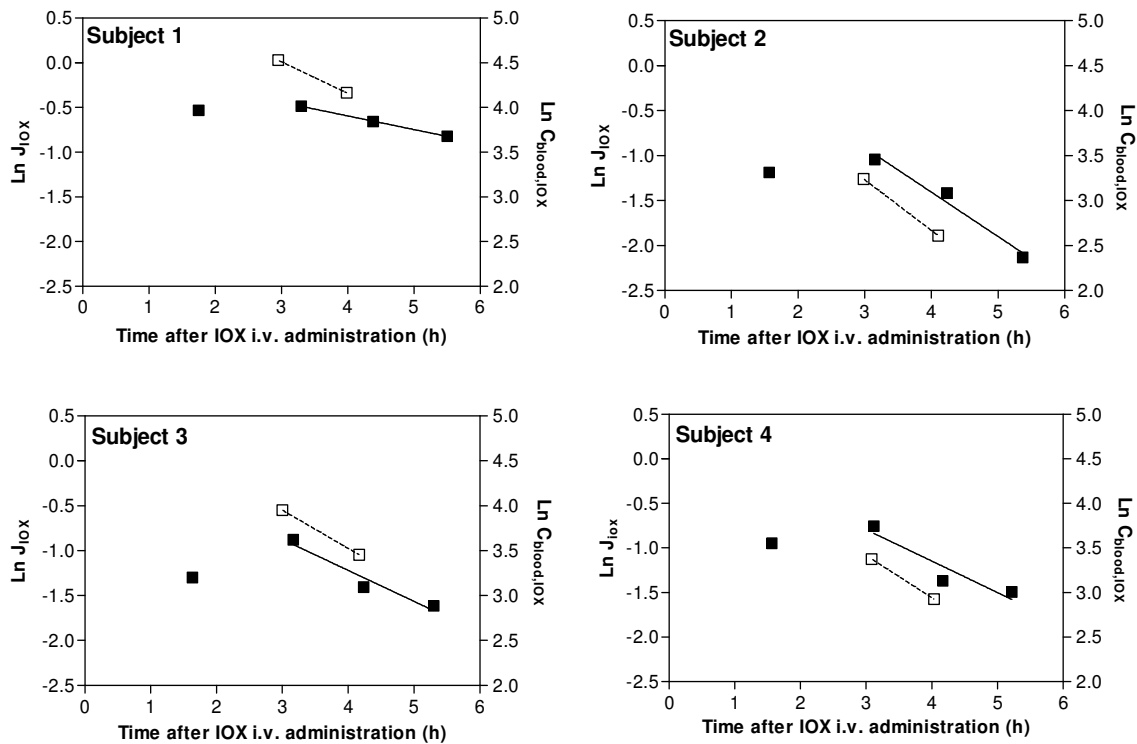
Figure 5

Figure 5: Iohexol transdermal iontophoretic extraction fluxes (J_{IOX}, $\mu g.h^{-1}$, filled squares) and blood concentrations (C_{IOX}, $\mu g.ml^{-1}$, open squares) as a function of time post-injection. The blood concentrations of iohexol are plotted at the exact times of blood sampling; whereas the transdermal extraction fluxes are plotted at the midpoint of the respective extraction interval. The regression lines are also shown for both sampling techniques. The current applied was 0.5 mA in all subjects except participant 3 for whom it was 0.3 mA.

The internal standard calibration of iohexol extraction fluxes using sodium resulted in a good correlation (r^2 = 0.88) between (J_{IOX}/J_{IS}) and the two measured blood concentrations (average κ'_{Na+} = 27.4 (±4.0) µl.mg^{-1}; Figure 6); individual κ'_{Na+} values, however, ranged between 3 and 60 µl.mg^{-1} (Table III). With potassium, the corresponding correlation was lower (r^2 = 0.49) with κ'_{K+} = 42.1 (±17.6) µl.mg^{-1}. As stated above, the selection of the best internal standard (or indeed the decision as to whether one is needed at all) awaits further study.
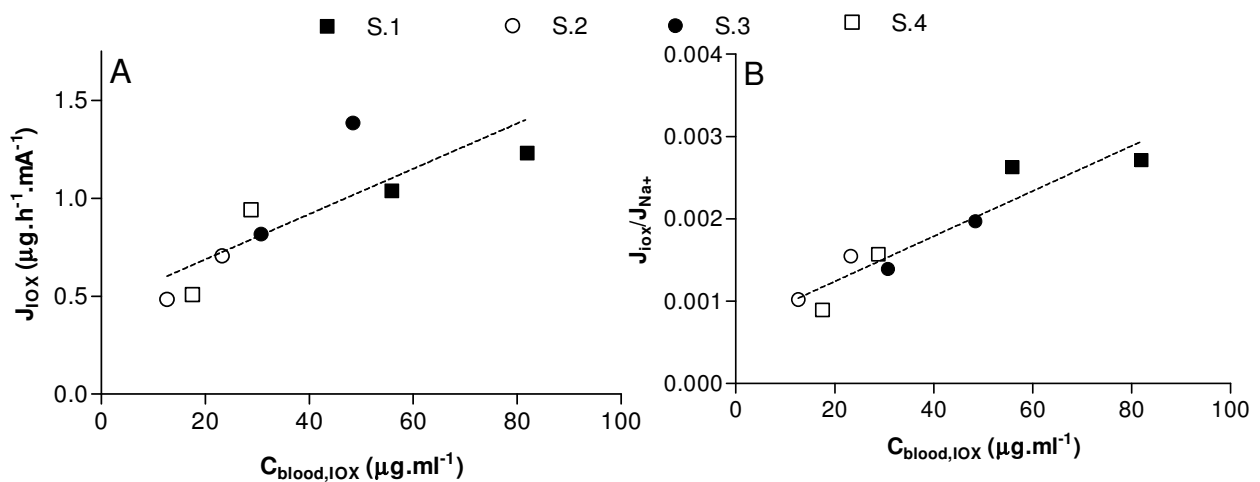
Figure 6

Figure 6: Iohexol extraction fluxes (J_{IOX}) (panel A) and ratio of iohexol and sodium extraction fluxes (J_{IOX}/J_{Na}) (panel B) as a function of the corresponding iohexol blood concentrations (C_{IOX}). The iohexol blood concentrations used in the regressions were corrected for the respective times of the middle of each iontophoretic period. Linear regression analysis provided the following slopes (±SE) for the relationships: (a) $J_{IOX} = \kappa_{IOX} \times C_{IOX}$ (r^2= 0.69); κ_{IOX} = 11.6 ± 3.2 µl.h^{-1}.mA^{-1}. (b) $J_{IOX}/J_{Na} = \kappa'_{Na} \times C_{IOX}$ (r^2=0.89); κ'_{Na} = 27.4 ± 4 µL.mg^{-1}. (c) $J_{IOX}/J_K = \kappa'_K.C_{blood,IOX}$ (r^2 0.49); κ'_K = 42.1 ± 17.6 µl.mg^{-1}.

The factor(s) causing variability in RI extraction efficiency (κ_{IOX}) *in vivo* cannot be unequivocally determined from the results of this pilot study. Previous investigations with the endogenous, uncharged analytes, glucose and urea, for which 'reservoirs' in the skin are known, have shown differing levels of inter- and intra-subject variability (18,37,40). The variability associated with glucose RI has never been satisfactorily explained, and whether the reason is primarily related to electro-osmosis or is specific to glucose, and its endogenous and ubiquitous presence in the body, has not been elucidated. In contrast, the RI of exogenously administered, lithium cations was efficient and quite constant across the cohort of individuals examined (19). There is at least a possibility, therefore, that the RI of iohexol, also an exogenous compound, will show low relative variability; only further work involving more frequent RI sampling, in a larger number of subjects with an improved sampling methodology, can address this issue.

It is pertinent at this point to compare the reverse iontophoretic extraction of iohexol and glucose, the only analyte for which a device, the Glucowatch Biographer®, based on RI extraction, has been developed and marketed (20,21,41). While the same core technology, (iontophoresis) and mechanism (electro-osmosis) underlie glucose and iohexol sampling, the two applications are distinct. GFR assessment takes place in a hospital setting and there is a finite, and very limited number of procedures required per patient; that is, there is no need for the ambulatory and continuous monitoring required by a diabetic patient. Thus, even if the method investigated here proved to be successful, there would be no need to develop an integrated sampling-analytical device such as the Glucowatch Biographer®; the investment required would not be justified by either the clinical need or the market size. The ultimate aim of this work was to render a current and effective hospital procedure less invasive to the fragile patient population in which it is used. It follows that a more realistic

approach to implement iohexol sampling might involve use of a simple (and ideally already approved and available) iontophoresis device (e.g., the Phoresor) in combination with disposable collection patches from which iohexol can be extracted and subsequently analysed in a local clinical laboratory.

Another factor, which differentiates the RI application to iohexol from that to glucose, is that the former is an exogenous compound that is administered on an acute basis for GFR assessment. It follows that there is no reservoir of iohexol in the skin, nor any chance that one can develop over time. In contrast, a reservoir of glucose exists in the skin that has to be 'emptied' before the RI-extracted amounts of the analyte are correlated with systemic glycaemia (18, 20,21).

A third key difference is that the estimation of GFR only requires knowledge of iohexol's terminal elimination rate constant (β) from the blood; absolute systemic concentrations are not needed. Therefore, as long as the value of β determined from the RI-extracted samples is the same as that assessed from blood sampling, then the relationship between the amounts of the marker drawn across the skin to those in the blood is unimportant. In contrast, for glucose monitoring, it is absolutely essential to be able to relate the RI-extracted amounts to blood glucose concentrations, and this is only possible (at present) with a finger-stick calibration procedure.

A final, more subtle difference between the uses of RI to sample glucose and iohexol, concerns the "lag-time" for changes in blood levels to be manifest in the RI-extracted samples. For glucose monitoring, the values reported by the Glucowatch follows changes in systemic concentrations with a delay estimated to be on the order of 15-20 minutes, a factor that needs to be recognised when blood levels change rapidly in response to food

ingestion and insulin administration (41). In the case of iohexol after an intravenous injection, the pharmacokinetics follow a 2-compartment model and 2-3 hours are required for the distribution phase to be complete and for the terminal elimination, which directly reflects renal clearance, to commence. RI sampling from this moment will therefore report faithfully on the patient's GFR as long as the kinetics of redistribution from the peripheral compartment are rapid, relative to renal clearance. While this specific information is not directly available, the fact that iohexol has proved to be a useful marker for GFR means that its elimination cannot be rate-limited by its redistribution (1,6,10,14).

Overall, the *in vivo* protocol was well tolerated; all four participants completed the trial and preferred RI monitoring over blood sampling. Tingling or itching sensations and erythema were similar to those previously reported (19, 22, 37). Feedback from participants and their parents included recommendations to improve the RI system to a more compact, integrated and fully-portable device. A perceived limitation of the RI approach is that iohexol administration remains an invasive procedure. Unfortunately, because of the MW (821.14 Da) and log P (-3.05) of the marker, neither transdermal nor oral administration of the dose required (1.3 g in this study) is feasible. It was also suggested that a less adhesive patch would decrease the discomfort associated with its removal and that the size of the patches used was too large for a paediatric subjects. These issues will need to be addressed before further clinical studies take place.

5. Conclusions

Iontophoresis can successfully track the subdermal concentration profiles of iohexol, a relatively large and neutral marker of glomerular filtration, both *in vitro* and *in vivo*. The

efficiency of extraction *in vivo* was well predicted by the *in vitro* model used. A pilot *in vivo* study identified key issues to be investigated further, including validation of the technique in a larger cohort and establishing the consistency of extraction efficiency within and between subjects. Practical issues, including the development of comfortable devices specifically designed for skin sampling in the paediatric population need to be addressed as well before larger *in vivo* studies can be undertaken.

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Table I: Reverse iontophoresis extraction efficiencies (mean ± SD, n = 4-12) for IOX (κ_{IOX}) and for ACM (κ_{ACM}) in "fixed-subdermal concentration experiments" and according to the equations [$J_{IOX} = \kappa_{IOX} \times C_{IOX}$] and [$J_{ACM} = \kappa_{ACM} \times C_{ACM}$]. J_{IOX} and J_{ACM} are the RI extraction fluxes and C_{IOX} and C_{ACM} are the subdermal concentrations for IOX and ACM, respectively. ACM but no IOX was present in the subdermal solution during the 3 hours pre-treatment which corresponds to the pre-2 to pre-0 hours sampling intervals.

	Iohexol					
	Time (h)					
	1	2	3	4	5	6
κ_{IOX} (μl.h^{-1})	0.28±0.11	0.81±0.28	1.29±0.39[a]	1.62±0.46[b]	1.83±0.47[c]	1.96±0.51[d]
κ_{IOX} + Pre-treat (μl.h^{-1})	0.54±0.22	1.57±0.42	2.01±0.42	2.24±0.38	2.36±0.35	2.34±0.37
	Acetaminophen					
	Time (h)					
	Pre-2	Pre-1	Pre-0			
κ_{ACM} (μl.h^{-1})	0.78±.031	1.96±0.67	2.60±0.66[a]			
	1	2	3	4	5	6
	3.07±0.97[b]	3.10±0.83[c]	3.21±0.74[d]	3.39±0.73	3.36±0.62	3.35±0.74

[a,b,c] and [d] indicate pairs of κ_{IOX} and κ_{ACM} significantly different (p<0.01) two-way repeated measures ANOVA followed by Bonferroni post-test.

Table II: *In vitro* kinetic parameters derived from the "pharmacokinetic profile simulation" extraction experiments using either iohexol subdermal concentration (C_{IOX}) or extraction fluxes (J_{IOX}) data. See Material and Methods for detailed explanation.

		$r^2 \geq$	K_e^a (h^{-1})	$C_{0,IOX}^b$ (mM)	Vd^c (ml)	Cl^d (ml.h^{-1})	Cl^e (ml.h^{-1})
		Mono exponential decline experiments					
No pre-treat	C_{IOX}	0.98	0.38 ± 0.05	0.29 ± 0.02	4.02 ± 0.28	1.51 ± 0.14	1.53 ± 0.1
	J_{IOX}	0.94	0.41 ± 0.03	0.36 ± 0.08	3.26 ± 0.63	1.34 ± 0.17	1.31 ± 0.2
With Pre-treat	C_{IOX}	0.99	0.28 ± 0.01	1.23 ± 0.05	3.71 ± 0.16	1.04 ± 0.05	1.04 ± 0.06
	J_{IOX}	0.90	0.25 ± 0.05	1.21 ± 0.18	3.89 ± 0.63	0.96 ± 0.05	0.96 ± 0.05

		$r^2 \geq$	Z_1 (mM)	K_1 (h^{-1})	Z_2 (mM)	K_2 (h^{-1})
		Two-phase decline experiment				
With Pre-treat	C_{IOX}	0.99	6.12 ± 0.16	0.80 ± 0.01	1.22 ± 0.27	0.27 ± 0.03
	J_{IOX}	0.97	6.18 ± 0.68	0.74 ± 0.02	1.37 ± 0.30	0.27 ± 0.07

[a]Determined from the slope.

[b]Determined by extrapolation to t = 0.

[c]Calculated from: $Vd = Dose/C_{0,IOX}$.

[d]Calculated using $Cl = K_e.Vd$.

[e]Calculated using: $Cl = Dose/AUC_0^\infty$

Table III: Terminal rate constants (β) estimated through blood and skin (best-fit ± SE) sampling for four paediatric patients and their corresponding efficiencies of extraction. The numbers in parentheses correspond to the ratio ($\beta_{blood}/\beta_{skin}$). See Material and Methods for detailed explanation.

Technique		Terminal rate constant (β, h^{-1})			
		S.1	S.2	S.3	S.4
Blood sampling		0.35	0.56	0.43	0.48
Skin sampling		0.15 ± 0.003 *(2.33)*	0.49 ± 0.08 *(1.14)*	0.35 ± 0.09 *(1.23)*	0.35 ± 0.14 *(1.37)*
Skin sampling: Normalised with Na^{+}		0.18 ± 0.09 *(1.94)*	0.51 ± 0.07 *(1.10)*	0.42 ± 0.06 *(1.02)*	0.27 ± 0.16 *(1.78)*
Skin sampling: Normalised with K^{+}		0.19 ± 0.03 *(1.84)*	0.47 ± 0.1 *(1.19)*	0.52 ± 0.07 *(0.83)*	0.33 ± 0.18 *(1.46)*
		Efficiency of extraction			
		S.1	S.2	S.3	S.4
K ($\mu L.h^{-1}.mA^{-1}$)	Individual value (n=2)	7.5	20.9	32.1	38.3
	All subjects (n=8)		11.6 ± 3.18		
κ'_{Na+} ($\mu L.mg^{-1}$)	Individual value (n=2)	3.3	49.5	32.3	59.9
	All subjects (n=8)		27.4 ± 4.04		
κ'_{K+} ($\mu L.mg^{-1}$)	Individual value (n=2)	25.7	95.8	131.6	194.2
	All subjects (n=8)		42.07 ± 17.6		