Evaluation de l'ionophorèse inversée comme méthode non-invasive pour le monitoring thérapeutique

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THÈSE

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Avant propos

Certains médicaments (tableau 1) présentent des différences individuelles considérables en terme d'efficacité et de toxicité. Ceci complique considérablement la définition d'une relation dose/effet (1). Le monitoring thérapeutique consiste à surveiller les effets d'un traitement dans le but de réduire les variabilités de la réponse thérapeutique (1-5). Cette variabilité dépendant à la fois des propriétés du principe actif (pharmacocinétique et pharmacodynamie) et de l'état clinique du patient (insuffisance hépatique, rénale, …) (6).

Dans le cas de médicaments présentant une marge thérapeutique étroite, ces disparités individuelles font qu'une posologie idéale chez certains patients peut être toxique ou inefficace chez d'autres (6,7). Dans ce cas précis le monitoring thérapeutique constitue un outil essentiel à la disposition du praticien pour individualiser le traitement. Lorsque les effets d'un traitement (thérapeutiques ou toxiques) ne sont pas aisément visibles ou quantifiables par le patient ou le praticien (6) (par exemple : traitements prophylactiques antiépileptiques, antiarythmiques, stabilisateurs de l'humeur, etc…), le monitoring permet de confirmer un bénéfice thérapeutique et d'éviter des effets secondaires (7). Le monitoring thérapeutique permet aussi de contrôler la compliance du

Tableau 1: Principes actifs couramment monitorisés.

Antalgiques, Antipyrétiques / Anti-inflammatoires
- Paracétamol
- Salicylés

Antibiotiques
- Aminosides
  - Amikacine
  - Gentamicine
  - Nétilmicine
  - Tobramycine
- Vancomycine

Antiépileptiques:
- Carbamazépine
- Ethosuximide
- Phénobarbital
- Phénytoïne
- Primidone
- Acide valproïque

Cytotoxiques
- Méthotrexate

Bronchodilatateurs
- Théophylline

Insuffisance cardiaque / Antiarythmiques
- Digitaliques
  - Digoxine
  - Digitoxine
- Antiarhythmiques
  - Disopyramide
  - Lidocaïne
  - Procaïnamide/NAPA
  - Quinidine

Immunosuppresseurs
- Ciclosporine A

Antidépresseurs / Stabilisateurs de l'humeur
- Antidépresseurs imipraminiques
  - Amitriptyline
  - Désipramine
  - Imipramine
  - Nortriptyline
- Lithium

patient au traitement, d'établir la cause d'échecs thérapeutiques ou de surveiller la modification d'un traitement (interférence médicamenteuse, médicament ayant une pharmacocinétique non linéaire, etc…) (6-8).

Dès lors que les concentrations systémiques du médicament sont en relation étroite avec les effets thérapeutiques et secondaires, ce contrôle est classiquement réalisé par prise de sang. La fréquence du monitoring dépend beaucoup de la situation clinique du patient (6). Un monitoring régulier est requis lors de l'établissement ou de la modification du traitement. Un contrôle périodique moins fréquent est généralement prescrit pour les patients traités sur le long terme. Néanmoins, un contrôle immédiat peut-être décidé en cas d'échecs thérapeutiques, d'effets secondaires, de modifications physiologiques (par exemple : maladie, grossesse) risquant de perturber la pharmacocinétique du médicament ou encore lorsqu'une non-compliance est suspectée.

Des méthodes moins invasives que la prise de sang offrent des bénéfices évidents : moins douloureuses du point de vue du patient, elles contribuent davantage au confort du patient lorsqu'un usage ambulatoire est possible. D'un point de vue médical, elles facilitent le contrôle chez les patients, diminuent les risques de complication (hématomes, infections,…) et rendent possible un monitoring plus fréquent permettant un meilleur suivi thérapeutique. Ces techniques non-invasives sont particulièrement utiles chez certaines populations (pédiatriques, gériatriques, soins intensifs, etc…) dont les facteurs physiologiques ou pathologiques perturbent la pharmacocinétique des médicaments et nécessiteraient par conséquent un monitoring plus fréquent (1,9-13). Pourtant, cet objectif reste trop souvent inaccessible par prise de sang pour diverses raisons : fréquence, volume de prélèvement, etc…(14). De plus le monitoring est souvent indispensable chez ces populations exprimant difficilement les effets bénéfiques ou secondaires d'un traitement. D'autant que l'information pharmacocinétique disponible reste limitée obligeant souvent le praticien à extrapoler les valeurs connues (15). Disposer d'outils non-invasifs favoriserait alors la réalisation d'études pharmacocinétiques dans ces populations sensibles (16-18).

Différentes stratégies non-invasives ont été proposées. Certaines techniques limitent le nombre de prélèvement par l'usage d'outils statistiques maximisant les informations obtenues d'un nombre limité de prélèvements (6,19). D'autres totalement non-invasives s'intéressent aux fluides et matrices biologiques alternatives (10,20,21). Dans le cas de l'urine, les cheveux et la sueur, ces applications se limitent à la détection de composés en toxicologie ou médecine légale (22-25). La salive fut sans doute l'alternative la plus étudiée à des fins quantitatives pour le monitoring thérapeutique (26-30). Cependant, au regard d'une grande variabilité inter-individuelle des fractions extraites et d'une forte sensibilité à de nombreux facteurs (pH, débit de sécrétion salivaire, etc …) son utilisation reste limitée (10,30).

La peau représente une alternative séduisante par sa surface importante et son accès facile, mais son excellente fonction barrière limite considérablement le transport des molécules polaires et chargées. Des études menées in-vitro et in-vivo, ont pourtant mis en évidence la diffusion transdermique de certains principes actifs (31-33). La lenteur du processus d'extraction (indistinctement diffusion passive et sudation) exige de concentrer le médicament dans un patch pendant plus de 10 h. Les résultats ont montré une grande variabilité de la diffusion passive transdermique, ce qui limite considérablement son utilisation pour un usage en monitoring thérapeutique (34,35). Diverses approches ont été proposées dans le but d'augmenter les quantités extraites (36). Ces méthodes perturbent la fonction barrière de la peau de manière plus ou moins transitoires et réversibles par un effet mécanique (sonophorèse, microaiguilles), électrique (ionophorèse, électroporation) ou chimique (36-41).

Ce projet à pour objectif d'évaluer les capacités de l'ionophorèse pour le monitoring non-invasif des médicaments au travers de la peau. Un faible courant électrique (< 0,5 mA/cm^2) appliqué à la surface de la peau permet de faciliter le passage transdermique des molécules (42). Parce que le champ électrique agit directement sur les molécules transportées et non sur la barrière cutanée, l'ionophorèse inversée offre un meilleur contrôle du transport et une moindre irritation de la peau que les autres techniques citées précédemment. L'ionophorèse est un processus actif qui permet à la fois l'administration et l'extraction de composés (42). L'ionophorèse inversée, qui permet l'extraction transdermique, fut développée intensivement pour le dosage de la glycémie, et plus récemment pour les dosages biologiques de la phenylalanine et de l'urée (43-47). Deux études *in-vitro* d'extraction de la clonidine, la théophylline et l'acide valproïque (45,48) ont mis en évidence le potentiel de la technique pour le monitoring thérapeutique.

Dans le but d'étudier les capacités de l'ionophorèse inversée pour le monitoring thérapeutique, deux principes actifs ont été choisis : la phénytoïne et le lithium. Puisqu'il est établi que l'extraction ionophorètique dépend, entre autre, des propriétés physico-chimiques des molécules, ces deux principes actifs sont très différents (Tableau 2) (49,50). La phénytoïne de taille moléculaire importante présente un caractère lipophile, une forte liaison aux protéines plasmatiques ainsi qu'une ionisation négative et partielle qui permet son transport par électrorépulsion et électroosmose. Le lithium est un cation de faible masse moléculaire, fortement mobile et quinze fois plus concentré dans le sang que la phénytoïne. Le lithium constitue l'un des meilleurs candidats pour un monitoring thérapeutique par ionophorèse.

Tableau 2 : Propriétés de la phénytoïne et du lithium

	Phénytoïne	Lithium
Classe thérapeutique	Anti-épileptique	Stabilisateur de l'humeur
Poids moléculaire (g/mol)	252	7
Lipophilie (Log P)	2.5	-
Marge thérapeutique (µM)	40-80	500-1400
Fraction libre	10%	100%
Charge (% ionisation)	Négative (12%) Neutre (88%)	Positive (100%)

Dans ce projet, les études menées *in vitro* et *in vivo*, (a) établissent la relation entre l'extraction ionophorètique et les concentrations subdermiques ou sériques. (b) Avec la phénytoïne illustrant le cas d'un principe actif fortement lié aux protéines plasmatiques, une étude *in vitro* démontre l'accès direct de l'ionophorèse à la fraction libre des principes actifs. (c) Avec le lithium, une étude évalue l'aptitude de la technique à mener des études pharmacocinétiques. (d) Toutes ces études font la preuve, in vitro et in-vivo, du concept d'auto-calibration de la méthode par un standard interne.

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Structure de la thèse

La thèse est structurée en deux parties. La première partie est une revue de la littérature sur le sujet de l'ionophorèse inversée pour le monitoring transdermique non-invasif.

Chapitre 1 : Dans un premier chapitre, ce manuscrit dresse un état de la technologie. Les mécanismes d'extraction de l'ionophorèse inversée, ses avantages et ses inconvénients, les applications et les récents progrès dont cette technique a fait l'objet sont décrits dans cette revue de la littérature.

Dans une seconde partie expérimentale, le manuscrit s'attache à évaluer les capacités de l'ionophorèse inversée pour le monitoring thérapeutique non-invasif. Deux médicaments, nécessitant une surveillance thérapeutique périodique, ont été choisis comme modèle pour cette étude.

Chapitre 2 : La phénytoïne illustre le cas d'un médicament difficilement extractible par ionophorèse transdermique et fortement lié aux protéines plasmatiques. Ce chapitre examine la relation de dépendance de l'extraction ionophorètique vis-à-vis des concentrations subdermiques (totale et libre) de phénytoïne.

Chapitre 3 : Dans ce chapitre, le lithium est utilisé comme modèle pour l'évaluation *in vitro* des aptitudes de l'ionophorèse inversée à réaliser non-invasivement des études pharmacocinétiques.

Chapitre 4 : Ce chapitre rend compte des résultats d'une étude menée chez des patients sous lithiothérapie. Cette étude examine la capacité de la technique à proposer une méthode alternative et non invasive pour la surveillance de la lithiémie chez les patients bipolaires.

Chapitre 5 : Dans ce dernier chapitre du manuscrit, les auteurs proposent une optimisation de la formulation des matrices d'extractions ionophorètiques en vue d'applications compatibles avec les contraintes des essais *in-vivo*.

Chapitre 1

L'ionophorèse inversée pour le monitoring transdermique non-invasif

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Résumé

Ce premier chapitre dresse un état de la technologie. L'ionophorèse consiste en l'application d'un faible courant électrique à la surface de la peau dans le but d'augmenter le transport transdermique des molécules polaires chargées et neutres. Le contrôle de l'électromigration et l'électroosmose, les deux principaux mécanismes de transport de l'ionophorèse, est rendu possible par la modification du courant appliqué et/ou de certains paramètres de formulation des compartiments récepteurs. Alors que cette approche a principalement été utilisée pour l'administration des médicaments par voie transdermique, l'ionophorèse inversée, permettant l'extraction de substances à la surface de la peau, a récemment fait l'objet d'efforts considérables. Cette revue de la littérature décrit les mécanismes d'extraction de l'ionophorèse inversée, ses avantages et ses inconvénients, les applications et les récents progrès dont cette technique a fait l'objet.

Mots-clés: Ionophorèse, Électromigration, Électroosmose, Surveillance de la glycémie, Monitoring thérapeutique

Reverse iontophoresis for non-invasive transdermal monitoring

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Abstract

Iontophoresis is the application of a small electric current to enhance the transport of both charged and polar, neutral compounds across the skin. Manipulation of either the total charge delivered and/or certain electrode formulation parameters allows control of electromigration and electroosmosis, the two principal mechanisms of transdermal iontophoresis. While the approach has been mainly used for transdermal drug delivery, "reverse iontophoresis", by which substances are extracted to the skin surface, has recently been the subject of considerable effort. Glucose monitoring has been extensively studied and other applications, including therapeutic drug monitoring, are contributing to the development of the technique. An internal standard calibration procedure may ultimately render this novel monitoring technique completely non-invasive.

Keywords: Iontophoresis, Electromigration, Electroosmosis, Glucose monitoring, Therapeutic drug monitoring

I. Introduction

Iontophoresis involves the application of a small and defined electrical current to the skin. This process causes increased molecular transport through the skin and has found application, therefore, in transdermal drug delivery [1]. The concept is not new, however; the basic mechanisms were clearly appreciated by Leduc [2,3] in 1900. Nevertheless, it is only now that approved iontophoretic drug delivery systems are finally reaching the market [4].

The symmetry of iontophoresis means that it also enables extraction of solute molecules from within the subdermal compartment to the skin surface. The potential exists, therefore, to use the technique for clinical chemistry without blood sampling. Applications that may be envisaged include general blood chemistry, glucose monitoring, the detection of diagnostic markers, and therapeutic drug monitoring [4]. The mechanism of extraction involves either electromigration of charged species to the electrode of opposite polarity, or electroosmosis of polar, neutral or zwitterionic, molecules to the cathode; hence for cations, both mechanisms are operative.

A number of excellent reviews on iontophoresis and its applications have been published [1,3,5-9]. Here, attention is focused on reverse iontophoresis and its applications in diagnosis and monitoring. After a brief consideration of the basics underlying electrotransport across the skin, a detailed evaluation of the relevant literature, and a perspective on the future, are presented.

II. Mechanisms of transport during reverse iontophoresis

A. Electromigration

Conventionally, in iontophoresis, a constant current is applied, such that the flow of electrons is translated into an ion flux across the skin. A power supply establishes the electric field that causes electrons to migrate in the "electrical" portion of the circuit and ions to flow in the "ionic" part (Figure 1). It follows that the number of electrons flowing through the "electrical" portion of the circuit is exactly balanced by the amount of ionic charge flowing through the skin [3].

The electromigration contribution to iontophoretic transport is a direct result of current application. Ionic transport proceeds

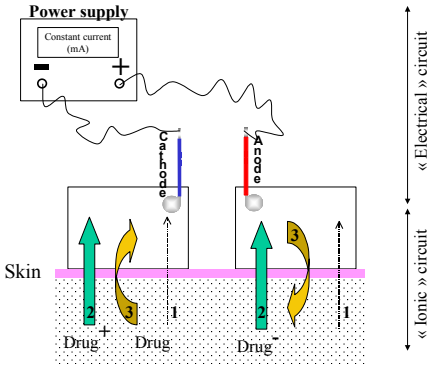

Figure 1: Reverse iontophoresis: a schematic diagram illustrating the experimental set-up. Constant current is delivered to the anode and the cathode from a power supply. Cationic and neutral substances are drawn towards the cathode by electromigration (2), electroosmosis (3) and (to a negligible extent, typically) passive diffusion (1). Anionic compounds are attracted into the anode chamber by electromigration (2), while convective solvent flow (3) opposes this phenomenon (again, passive diffusion (1) is negligible).

through the skin to maintain electroneutrality [1,3,7]. Faraday's law applies to steady-state transport and relates the number of ions crossing the membrane to the electric current, the time of current passage and the charge per ion [1,3]:

$$M_i = \frac{T.i_i}{F.z_i} \qquad \text{Equation 1}$$

where M_i is the number of moles of the 'i' ion, T is the time (s), z_i is the valence, F is Faraday's constant (96 487 $C.mol^{-1}$), and i_i the current (A) carried by the i^{th} species.

Given that usually there is more than one ion moving across the barrier, the total number of moles transported (M) by the total current flowing (I) is given by:

$$M = \sum_i M_i = \frac{T}{F}\sum_i \frac{i_i}{z_i} \qquad \text{Equation 2}$$

$$\text{where } I = \sum_i i_i \qquad \text{Equation 3}$$

This leads to the concept of an ion transport number (efficiency of transport) which is the fraction of the total charge that it transports:

$$t_i = \frac{i_i}{I} \qquad \text{Equation 4}$$

It follows that Equation 1 may be rewritten:

$$M_i = \frac{t_i.I.T}{F.z_i} \qquad \text{Equation 5}$$

With respect to the subject of this review, the experimentally measured extraction flux (J_i, $mol.s^{-1}$) is defined by the ratio of number of moles transported (M_i) to the sampling time (T) (i.e., the duration of reverse iontophoresis):

$$J_i = \frac{M_i}{T} = \frac{t_i}{F.z_i}.I \qquad \text{Equation 6}$$

Equation 5 shows that iontophoretic extraction is determined by the intensity of current, the time of iontophoresis, the charge and the transport number of the ion of interest. Current intensity (I) is directly and easily controlled by the power supply but is limited, for practical purposes *in vivo*, to no more than 0.5mA/cm² [6]. The time of each extraction period must be sufficiently long to ensure that enough analyte is available for detection but not so long that clinically significant changes in the systemic concentration may have occurred. In any case, it must be recognized that reverse iontophoresis can only provide an estimation of the average level of the analyte in the body during the sampling period.

The charge (z_i) of the extracted ion is dictated by its molecular structure, and determines the polarity of the electrode at which sampling/analysis will be performed. The transport number, however, is difficult to estimate theoretically as it depends on the other ions contributing to the transport of charge across the skin. When an electric field is established across a membrane, ions on either side will migrate in the direction dictated by their charge. The speed of migration of an ion is determined by its physicochemical characteristics and the properties of the media through which the ion is moving [3,7,8,10]. The sum of the individual ionic charges flowing across the skin must equal the number of electrons "delivered" by the power supply; in other words, there is "competition" among all the ions present to carry the charge.

The transport number of the ion of interest (the i^{th} ion) may also be expressed as follows:

$$t_i = \frac{c_i.z_i.u_i}{\sum_{j=1}^{n}(c_j.z_j.u_j)} \qquad \text{Equation 7}$$

where c_j is the concentration ($mol.cm^{-3}$), z_j the valence, and u_j the mobility ($cm^2.s^{-1}.V^{-1}$) of each of the "n" ions in the system [7].

Logically, the transport number depends on concentration (i.e., the available amount of a particular ion to participate in carrying charge across the skin). Likewise, it makes sense that ions, which are more mobile, will play a greater role in the movement of charge through the barrier. Note, however, that the relevant values of concentration (c) and mobility (u) are those inside the skin, rendering their estimation tricky at best and emphasizing the limitations of Equation 7 as a predictive tool.

In reverse iontophoresis, the concentration of the analyte is the variable of interest and will depend (in the case of a drug being monitored, for example) on the dosage regimen and the relevant pharmacokinetics. Furthermore, with respect to electromigration, only the ionized fraction of the analyte is extractable and this will depend on the relevant pK_a. Similarly, for analytes that are bound to proteins, it is clear that only the free fraction can significantly contribute to charge transport across the skin. Figure 2 illustrates this point for phenytoin, a drug that is normally ~90% bound to albumin. As far as ionic mobility is concerned, regardless of the medium through which transport is occurring, an inverse dependence upon molecular size can be confirmed (and this is another reason, of course, why only unbound substances are extractable by reverse iontophoresis). In summary, therefore, it can be concluded that an ion can function as a major charge carrier if it is small, fully charged, at high concentration, and not significantly protein-bound. Additionally, and ideally, "competing" ions are minimized, a situation not practically realizable for reverse iontophoresis where the major charge carriers are Na^+ and Cl^-.

Lastly, it is worth noting that the transport number is a "formulation-dependent" parameter applicable for a given set of conditions. The transport number t_i can be determined experimentally; for example, by dividing the total amount of the ion transported by the total charge delivered (using Faraday's law, Equation 1), or from the gradient of a graph of ion flux versus current intensity (as in Equation 6) [11].

B. Electroosmosis

At physiologic pH, the skin is negatively-charged and cation-permselective. When an electric field is imposed across this type of membrane, there is convective or electroosmotic solvent flow induced in the anode to the cathode direction (i.e., in the direction of counter-ion migration) [5,12]. This stream of solvent carries along with it dissolved solutes thereby enhancing the transport of neutral and, especially, polar molecules. Electroosmosis thus reinforces the transport of cations while acting against that of anions.

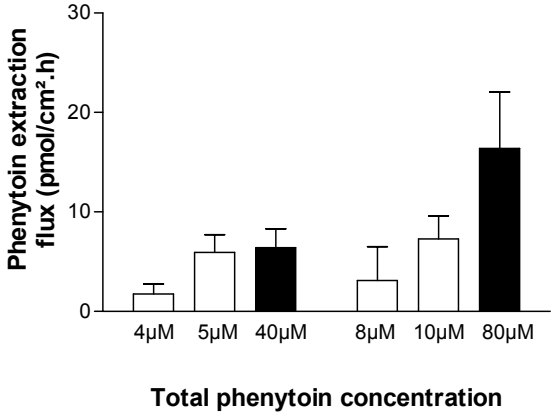

Figure 2: In vitro reverse iontophoresis fluxes of phenytoin at the anode illustrate that only free drug is extracted. The open bars (mean ± SD) indicate extraction of drug from a subdermal compartment that did not contain albumin. The filled bars represent data obtained when human serum albumin was present at 44 g/l. The results are consistent with a free fraction of phenytoin of approximately 0.1. Data redrawn from [25].

Important characteristics of this mechanism of electrotransport are, first, that the solvent volume flow (J_{vs}, volume.time^{-1}.area^{-1}) is proportional to the potential gradient across the skin [12,13] and, second, that the electroosmotic flux of solute (J_i) is independent of molecular size (at least as long as the solute diameter does not approach that of the transport pathway) [5,12]. The relationship between the molar flux (J_i) of the solute "i" and its molar concentration (c_i) is given by [14]:

$$J_i = J_{VS}.c_i \qquad \text{Equation 8}$$

In an elegant series of experiments, J_{vs} during iontophoresis [14] was determined to be 6-19 µl.h^{-1}.mA^{-1}.

In addition to the current density, the pH and the ionic strength are electrode formulation parameters that may modulate electroosmosis [15]. Modifying the pH on either side of the skin can change the charge on the membrane and hence its permselectivity. Practically speaking, only the surface pH can be altered *in vivo*, of course. Figure 3 illustrates that cathodic extraction of phenylalanine is enhanced by as high a pH as can be feasibly maintained in contact with the skin surface [16]; in contrast, an acidic pH in the cathode chamber significantly impairs electroosmosis towards the electrode [15], while favoring extraction in the opposite direction, presumably due to a degree of neutralization of the fixed charge on the skin.

For cathodal extraction, electroosmotic flow is increased by lowering the ionic strength of the electrode formulation [15,16]. This phenomenon is less obvious for anodal extraction [15]. However, it should be remembered that a finite level of electrolyte must be present in the electrode chambers (particularly at the anode) to support the Ag/AgCl electrochemistry.

It has also been found that an anode formulation with $CaCl_2$ or $MgCl_2$, instead of NaCl, increased electroosmotic flow from beneath the skin surface towards the anode [17]. Shielding of the net negative charge on the skin is a possible mechanism for this observation. In contrast, in the cathode chamber, to which electroosmosis predominates, enhanced solvent flow was achieved by formulating the electrode bathing solution with Ca^{++} binding agents (calcein, heparin, or EDTA) presumably exposing a greater negative charge on the skin [16,17].

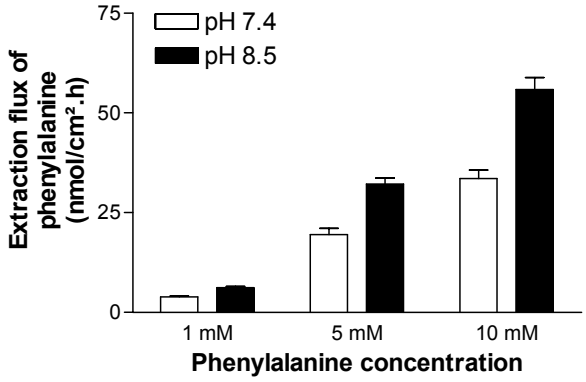

Figure 3: Electroosmotic extraction of phenylalanine as a function of (a) the subdermal concentration of the amino acid, and (b) the pH of the cathodal formulation. Reverse iontophoretic fluxes increased linearly with analyte concentration, and were significantly higher at pH 8.5 than at pH 7.4. Data redrawn from [16].

C. The dominant mechanism: electromigration or electroosmosis?

For small mobile ions, electromigration is clearly the principal mechanism; similarly, for neutral, polar substances, electroosmosis dominates as there is no electromigration possible from the electrode. Both mechanisms of electrotransport depend upon the applied current [10,18], with the effect being less marked for electroosmosis [13,19]. As the size of an ion increases, its mobility is reduced and electromigration is compromised. For cations, this means that the dominant mechanism switches from electromigration to electroosmosis with increasing molecular size [20]; for anions, on the other hand, the two contributions will ultimately self-cancel and no transport will be observed. A weak acid, therefore, which is only partially ionized at physiological pH, may be more easily extracted as its neutral form to the cathode (e.g. theophylline [21,22]).

III. Advantages and limitations of reverse iontophoresis

Noninvasive sampling methodologies are of obvious benefit to all patients for at least the following reasons: more information is obtainable as sampling can be performed more frequently, decreased pain and discomfort (i.e., better compliance), decreased risk of infection, potential for home-monitoring, etc.

Furthermore, there are special populations for whom noninvasive diagnosis and monitoring would be particularly useful: patients who are repetitively subjected to invasive blood withdrawal procedures, patients who over- or under-respond to standard therapeutic regimens, subjects who are least able to tolerate, recognize or communicate problems with unexpected drug effects, and those who, for whatever reason, are either over- or under-dosed. Such patient populations include the “critically” ill under intensive care, cancer and AIDS sufferers, pregnant women, those displaying unusual pharmacokinetics, patients receiving simultaneous, multi-drug dosing regimens (e.g., the elderly) and, of obvious special concern, are pediatric patients, for whom the need for noninvasive diagnosis and monitoring is particularly acute.

Even though reverse iontophoresis is much more efficient and reproducible than passive extraction, the quantities of analyte obtained at the skin surface are necessarily small. Dilution factors are likely to fall in the 10 to 100-fold range, or higher. Thus, analytical chemistry demands are significant and sampling periods may be so long, as a result, that changes in systemic concentration can occur. While this would be unacceptable in the case of glucose, for therapeutic drug monitoring at 'steady-state' the problem is less important (i.e., continuous monitoring is unnecessary, and a prolonged sampling time to obtain an average measure of concentration would be reasonable). Thus, an iontophoretic patch could be worn for a few hours at home, for example, and then sent to the clinical chemistry laboratory. Such an “off-line” analysis and quantification would be acceptable, and a sampling device simpler and less expensive to design.

An important limitation occurs when the skin accumulates the analyte of interest such that the initial extraction sample contains mostly information about this local 'reservoir' (this is the case for glucose [21] and lithium [23], for example). A “warm-up” period is necessary, therefore, before readings reflective of systemic levels are obtained.

It is also true that the reverse iontophoretic flux does not reach a constant, 'steady-state' rate instantaneously [24,25] – the time to do so depends on the molecule of interest, and the dominant mechanism of electrotransport. However, whether this limitation is significant has not been completely established; for certain analytes, an acceptable correlation between extraction flux and subdermal

concentration is obtained prior to steady-state as well.

An additional, and significant, limitation is that reverse iontophoresis will simply not work for molecules with particular physicochemical properties. Specifically, proteins, for example, are simply too large to be extracted in amounts that are quantifiable. Extremely lipophilic compounds, with extremely small aqueous solubilities, will also be undetectable; unfortunately, cholesterol falls into this category.

IV. Case studies

In 1954, reverse iontophoresis was first applied to the extraction of sodium and potassium ions [26]. Experiments were performed *in vivo*, in man, using a metal plate as the electrode. A current density of 0.5 mA/cm² was applied over a skin surface of 8.3 cm² for 5 minutes or longer. In a total of nearly 100 subjects, it was shown that age, gender, measurement time, measurement site and ambient temperature did not significantly affect the amounts of the cations extracted (Table 1). However, in the longer duration experiments, skin "damage" was observed due to the fact that the pH of the cathodal solution increased from between 6 and 7 to nearly 11. Clearly, therefore, electrolysis of water was taking place at the bare metal electrode and, as a result, it became sensible to henceforth use electrochemically reversible electrodes (e.g. Ag/AgCl) in iontophoresis studies [27].

The practical potential of reverse iontophoresis was appreciated much later (in 1989) when it was demonstrated that the amount of a substance extracted across the skin in this way was linearly related to the subdermal (and, by extrapolation, the systemic) concentration [21]. This relationship was shown for clonidine, theophylline and glucose; that is, for a more or less fully charged cation, for a partially charged anion, and for a neutral polar molecule (Figure 4).

Table 1: *In vivo* reverse iontophoretic extraction of potassium and sodium ions as a function of different parameters. The study group comprised 98 human volunteers. Data from [26].

	Potassium (µmol/h)	Sodium (µmol/h)	Ratio
Measurement time			
(10 measures)	1.8 ± 0.3	4.3 ± 0.5	2.4 ± 0.6
Measurement site			
Volar surface forearm	1.8 ± 0.4	4.3 ± 0.8	2.4 ± 0.4
Back of lower leg	1.6 ± 0.5	4.2 ± 0.8	2.7 ± 0.5
Upper part of abdomen	1.9 ± 0.4	4.5 ± 1.0	2.4 ± 0.5
Ambient temprature			
32-33°C	1.8 ± 0.3	4.3 ± 0.5	2.4 ± 0.4
21°C	1.7 ± 0.4	4.4 ± 0.6	2.5 ± 0.4
Gender			
Male	1.7 ± 0.5	4.3 ± 0.8	2.5 ± 0.3
Female	1.7 ± 0.5	4.2 ± 0.6	2.5 ± 0.3
Age			
<24 years	1.8 ± 0.5	4.2 ± 0.7	2.3 ± 0.5
25-49 years	1.7 ± 0.3	4.3 ± 0.5	2.6 ± 0.3
>50 years	1.6 ± 0.3	4.4 ± 0.5	2.7 ± 0.4

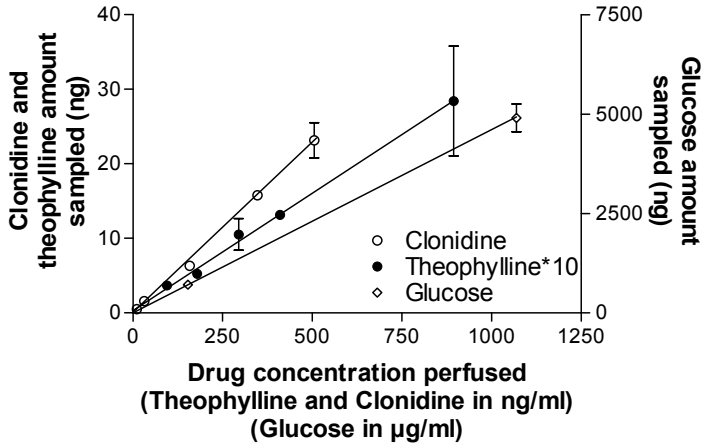

Figure 4: *In vitro* iontophoretic sampling of clonidine, theophylline and glucose. The amounts of the three compounds extracted across hairless mouse skin were linearly correlated with their subdermal drug concentrations. Data redrawn from [21].

Subsequently, attention became focused upon glucose. Obviously, the availability of a noninvasive tool with which to monitor blood sugar in diabetics would be of immense medical benefit. The conventional, "finger-stick" method, while precise and effective, is rarely used with sufficient frequency to reduce or avoid either hypo or hyper-glycemic events, despite compelling evidence that such an approach can significantly impact the chronic progression of the disease [28]. Reverse iontophoresis experiments *in vitro* [29] and initial *in vivo* studies in non-diabetic subjects [30] established proof-of-concept, and led to the commercial development of an integrated device (the Glucowatch Biographer® [31,32]) which is able to extract glucose iontophoretically across the skin and then assay sugar *in situ* with an on-board amperometric biosensor (Figure 5). The mechanism of electrotransport of glucose is electroosmosis, meaning that, during each

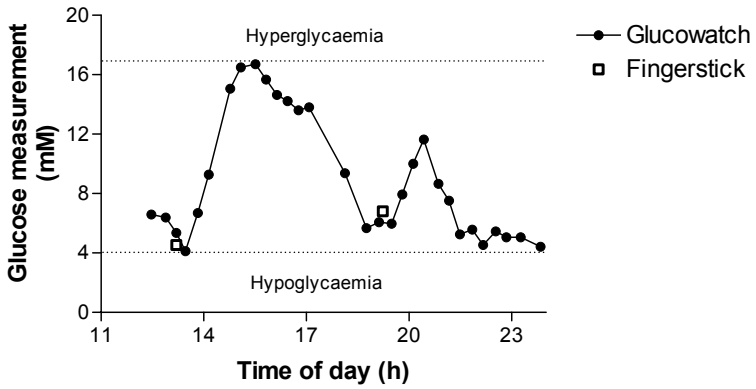

Figure 5: Continuous glucose monitoring in vivo with the Glucowatch Biographer® over a 12-hour period (closed circles) compared with the typical information available to a diabetic from two "finger-stick" measurements (open squares) pre-lunch and pre-dinner. Data redrawn from [32].

sampling period (20 minutes, initially, a shorter time in the G2 version of the device), the amount of analyte to be detected is very small. An exquisitely sensitive analytical method is therefore required, and involves a highly optimized adaptation of the Pt-glucose oxidase sensor [33].

A significant quantity of data has now been published to illustrate the efficiency of this reverse iontophoresis technology to track changes in the blood sugar levels of diabetics over the entire range of glycemia [32,34-39]. The quality of this information led to the Glucowatch being approved for use in adults by the U.S. Food and Drug Administration in 2001. Additional work in children (7-17 years) allowed this approval to be extended to juvenile diabetics the following year [40].

The long-term use and usefulness of the Glucowatch remain to be seen; nevertheless, there can be little doubt that this first truly noninvasive approach to the monitoring of blood sugar has made a paradigm shift in the field. It should be said that limitations of the approach are apparent, not least the lenghtly, 2-3 hour, warm-up time before measurements can be made (due to the need to empty a glucose reservoir in the skin) and the fact that a "finger-stick" blood measurement is essential to calibrate the device.

Other applications of reverse iontophoresis can be divided into diagnosis/monitoring and therapeutic drug monitoring. An innovative concept was to use the approach as a diagnostic tool for cutaneous inflammation [41]. Prostaglandin E2 (PGE_2) was monitored in response to the transdermal delivery of irritant drugs. It was hypothesized and shown that low-level iontophoresis (0.05 mA/cm² over 2 cm²) of saline did not by itself provoke an increased production of inflammatory markers *in vivo*, in the hairless guinea pig. Subsequently, potentially irritant drugs (chlorpromazine, chloroquine, promazine, tetracaine and metoclopramide) were administered iontophoretically (Figure 6). Then, the anodal extraction of PGE_2 from the site of drug administration was monitored and compared to the saline control. Significant increases were observed that correlated well with more classic determinations of irritation (e.g. the Draize test, lesion score).

The reverse iontophoretic extraction of phenylalanine has also been demonstrated [16]. In phenylketonuria, a severe metabolic disease, the enzyme which biotransforms phenylalanine is missing. Early detection of the disease and subsequent control of the diet are therefore essential. Children with the disease are frequently monitored via blood samples and a noninvasive approach would therefore be of interest. Phenylalanine is zwitterionic at physiologic pH and is therefore extracted during reverse iontophoresis by an electroosmotic mechanism. Like glucose, it has been shown that the amounts detected at the

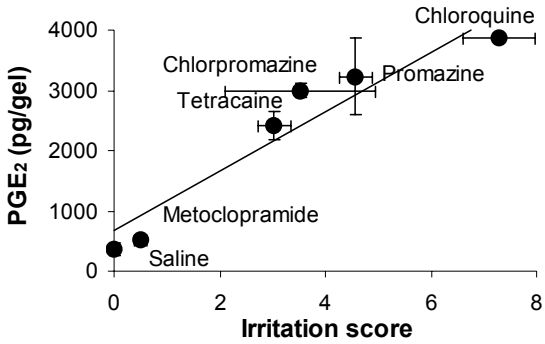

Figure 6: Correlation between amount (in pg) of PGE2 recovered in the gel and irritation (r^2=0.96). Iontophoretic systems containing 100 mM drug were applied at 50 µA/cm² for 24h. Graph redrawn from [41].

cathode are proportional to the subdermal concentrations (Figure 3). However, phenylalanine systemic concentrations, even in phenylketonuria, are much less than typical glucose levels in diabetics. It follows that the analytical chemistry challenge for the monitoring of phenylalanine via reverse iontophoresis is considerable. On the other hand it should be said that continuous and frequent monitoring, as performed by the Glucowatch®, is not necessary for sufferers of phenylketonuria; a device that simply collects the sample (once a day or once a week, for example), which is subsequently sent for analysis at a central analytical laboratory, would be perfectly acceptable.

Very recently, the reverse iontophoretic extraction of urea has been performed in 17 patients (21-35 years) with impaired kidney function [42]. Urea was extracted by electroosmosis to the cathode by current application for 5 minutes. The extracted amounts correlated well with urea levels in the blood (r^2=0.88). A logical application of this approach is to determine when dialysis should be performed in pediatric patients with kidney disease. A proof-of-concept study was subsequently conducted in six juvenile subjects (aged 9-16 years) for whom it was clearly shown that the amounts of urea extractable pre- and post-dialysis were quite different.

Therapeutic drug monitoring applications of reverse iontophoresis have recently attracted heightened interest. The potential of the approach was first explored using caffeine and theophylline in a model designed to mimic the developing cutaneous barrier in a premature neonate [22]. While the idea appears feasible for full-term infants, whose stratum corneum performs as well as that of an adult, the technique is less satisfactory when the barrier is impaired (as is the case, of course, for premature babies). The problem is caused by the fact that, superimposed upon the electrotransport of the target analyte being extracted by reverse iontophoresis, there is a significant passive transport which confounds straightforward interpretation of the data. Nevertheless, the noninvasive nature of the technique implies that it may have other useful applications in the case of the sick neonate, at least when skin barrier function is intact.

An important consideration when assessing the feasibility of reverse iontophoresis for therapeutic drug monitoring is the degree to which the compound of interest is protein-bound. Logically, only the free drug is electrotransported across the skin as the protein-bound form is too large to be extracted. This issue has been addressed with two anti-convulsant drugs, valproic acid [24] and phenytoin [25], approximately 90% of which are typically bound to plasma proteins. Reverse iontophoresis extraction of both drugs in a concentration dependent fashion was demonstrated over a wide-range encompassing those free levels observed in patients undergoing treatment. Valproate was extracted to the anode, while phenytoin (pKa=8.3) was recovered at both anode (the ionized fraction of the drug being attracted by electromigration) and cathode (the neutral form being carried by electroosmosis). When the level of protein was reduced in subdermal compartment, the amount of drug extracted was increased consistent with the rise of the free drug level (Figure 7). Equally, when monitoring phenytoin at a fixed subdermal protein level, introduction of valproate led logically to an increase in the free amount of the first drug and a higher rate of extraction due to the impact of competitive binding. The sensitivity of the method to respond to changes in free drug concentration in this way supports its potential usefulness for monitoring substances with a narrow therapeutic window. On the other hand, it must be recognized that, for lipophilic drugs like valproic acid and phenytoin, the free systemic concentrations are quite low (50 - 105 μM and 4 - 8 μM, respectively) and the amounts extracted by reverse iontophoresis are extremely small

Phenytoin	80 µM	40 to 80 µM	80 µM
Albumin	44 to 22 g/l	44 g/l	44 g/l
Valproate	-	-	0 to 542 µM
Free phenytoin	8 to 16 µM	4 to 8 µM	9 to 14 µM

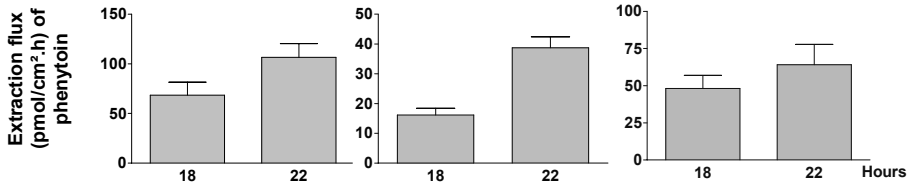

Figure 7: Monitoring of free phenytoin by reverse iontophoresis. The impact of (a) changing albumin concentration (1st panel), (b) changing drug concentration (2nd panel), and (c) addition of a competing drug, valproate (3rd panel), is illustrated. Data from [25].

(indeed, the *in vitro* experiments described above were performed with radiolabelled drugs). Once more, the analytical challenge *in vivo* will be very demanding.

With lithium, a drug used to treat bipolar disorders, on the other hand, the analytical chemistry problem is much less severe. First of all, as a small, non-protein bound cation, Li^+ is reverse iontophoretically extracted much more efficiently than the aforementioned anti-convulsant drugs [43]. Second, the effective plasma concentrations are much higher for lithium such that the amounts detected at the skin surface can be assayed with existing technology. *In vitro*, the linearity and rapidity of Li^+ extraction from a physiological buffer was simply demonstrated (Figure 8); when the concentration of Li^+ in the sub-dermal compartment was varied over time, to simulate a pharmacokinetic profile, the extraction profile closely followed the "absorption" and "elimination" phases of the curve. Subsequently, an *in vivo* study [23] in patients being treated with lithium has shown the potential of reverse iontophoresis to provide a useful clinical tool. The reverse iontophoretic extraction fluxes were extremely well-correlated with the corresponding plasma concentrations.

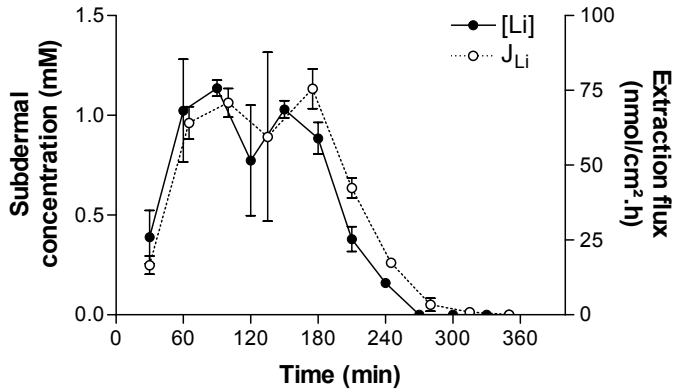

Figure 8: Monitoring of subdermal lithium concentration changes. Continuous line is the subdermal lithium concentration (mM). Dotted line is the cathodal extraction flux (nmol/cm².h). Data taken from [43].

V. Optimization

To expand the range of reverse iontophoresis applications, and improve on the existing technology, three strategies present themselves immediately for consideration.

Most obviously, an improvement in analytical sensitivity is desirable. The lower the extracted amount that can be detected, the larger the number of potential candidates for the technique, and the shorter the time of sampling (and, hence, the lower the total charge passed across the skin). Further discussion of this point, however, is outside the scope of this review.

Second, we may anticipate that optimization of the iontophoretic conditions will lead to maximization of the extracted amount. Thus, choosing the right current, current density, current profile and "acceptor" phase for the extracted analyte may be expected to improve the extraction efficiency. It is well-established that iontophoretic transport is directly proportional to the current and to the time of current application. In practice, however, there are limits to which these parameters can be increased. In terms of current density, it is generally agreed that 0.5 mA/cm² is the maximum tolerable in man [6]. It follows that the total current can be increased by increasing the surface area extracted. But, if one maintains the current density fixed, even under these conditions, the degree of sensation experienced by the subject increases with treated area, presumably because a greater number of dermal nociceptors are activated [30]. There are at least two other difficulties associated with increasing the total current and the area of skin over which reverse iontophoresis extraction is performed: (i) more current means that the (typically) Ag/AgCl electrodes used must be coated with an augmented larger of AgCl to ensure that the correct electrochemistry operates throughout the sampling period, and (ii) more area implies a greater volume into which the sample is collected, and this may place more demands on the analytical method. While it has been suggested that iontophoresis-induced reddening of skin can be reduced by 'pulsing' the current on/off at different frequencies [15], the practical result is that the impact of irritation is not proven. In addition, as the extraction is much less efficient during the 'off' period, the total time for sampling has to be increased so that the total desired current can be passed. Alternating current shows no benefits whatsoever; however, switching electrode polarity at the end of each sampling period, as is done in the Glucowatch®, has the distinct advantage of allowing regeneration of the Ag/AgCl electrodes [44].

The third strategy is a method to avoid the present necessity to calibrate reverse iontophoretic extraction of an analyte with a blood sample. The amount of the compound of interest recovered at the skin surface is diluted in a certain volume of "acceptor" fluid. The concentration therein depends on the efficiency of extraction (the analyte's transport number for an ion, the electroosmotic flow for a neutral species) and the volume of the "acceptor" solution. In the case of the Glucowatch®, for example, in each sampling period, the electroosmotic flow of less than 1µl is diluted into a volume of 400µl [44]; that is, a three order of magnitude dilution of the glucose. Calibration is therefore essential to relate the amount of sugar extracted to the blood concentration. The concept of an "internal standard" was initially proposed in 1993 [45] and has recently been significantly refined and reduced to practice [24]. The idea is as follows: reverse iontophoresis extracts numerous compounds at the same time; i.e., the process is non-specific and is only rendered specific for a particular compound by the use of a selective and precise assay. Suppose now that, in addition to the chosen target analyte (A), a second substance is also specifically analyzed in each sampling period. Suppose, further, that the blood concentration of this second molecule (IS) is effectively constant. It

follows that there should be a proportionality between the measured extraction flux ratio of A and IS (J_A/J_{IS}) and the ratio of their subdermal, or blood, concentrations (C_A/C_{IS}):

$$\frac{J_A}{J_{IS}} = K.\frac{C_A}{C_{IS}} \qquad \text{Equation 9}$$

It follows that, given C_{IS} is fixed and (presumably) known:

$$C_A = \frac{C_{IS}}{K}.\frac{J_A}{J_{IS}} \qquad \text{Equation 10}$$

Thus, if the proportionality constant K can be determined and shown to be invariant in a subject population, then an experimental determination of J_A/J_{IS}, together with the known, 'constant' term (C_{IS}/K), allows C_A to be found without the need for blood sampling. The success of this idea rests on the independence of the iontophoretic transport of the analyte and the internal standard. In other words, it is important that a change in the transport number of A (due, for example, to a fall in its systemic concentration) is not compensated by an increase in that of IS. In this case, the validity of Equation 9 breaks down. When the concept was proposed initially [45], the design of the experiments led to exactly this violation, with the result that the method could not be validated. Recently, however, the principle has been re-visited [24], the experimental test designed more carefully, and the technique has been shown to work. The first demonstrated success involved the reverse iontophoretic extraction of valproate using glutamic acid as an anionic internal standard [24]. Although the concentration of glutamate *in vivo* is not sufficiently constant for this amino acid to be considered as a practical internal standard, it served perfectly to prove the concept in this study. It was shown that (i) the extraction flux of valproate varied linearly with its subdermal concentration, (ii) the extraction flux of glutamate remained constant as the valproate concentration fluctuated, and (iii) the ratio of the valproate to glutamate extraction fluxes was proportional both to their subdermal concentration ratio (Equation 9) and, as the glutamate concentration was fixed, to the valproate concentration. The approach allowed experimental variability to be reduced and permitted the subdermal valproate to be found even before the iontophoretic transport achieved 'steady-state'.

Subsequently, similar success was achieved with the Li^+/Na^+(analyte/internal standard) couple (Figure 9). The sodium ion is a useful and practical internal standard due to the fact that the concentration of NaCl *in vivo* does not vary outside the range of 125-145 mM (and typically remains within a much narrower window); Na^+ is also the major charge carrier in iontophoresis in the outward direction towards the cathode (much as Cl^- performs the same function towards the anode). *In vivo* measurements, in patients, confirmed the constancy of the proportionality constant K in Equation 9. However, while *in vitro* experiments [46] indicated that Na^+ may prove a valid internal standard for glucose as well, a subsequent *in vivo* study revealed that electroosmotic flow is a much more sensitive phenomenon and can vary by nearly a factor of ten even while the electromigrative flux of Na^+ remains unchanged. It follows that it will be necessary to identify an electroosmotically-extracted internal standard for glucose in order to avoid the need for blood sampling.

VI. Conclusion

Recent progress in reverse iontophoresis confirms its considerable potential. The approval and commercialization of the Glucowatch® represents an important milestone for the technology as the first truly noninvasive monitoring device for diabetics. The value of the method is readily appreciated, furthermore, not only as a research tool but also as a practical means by which to improve the quality of care (and life) in patient populations for which repetitive blood sampling represents a significant burden:

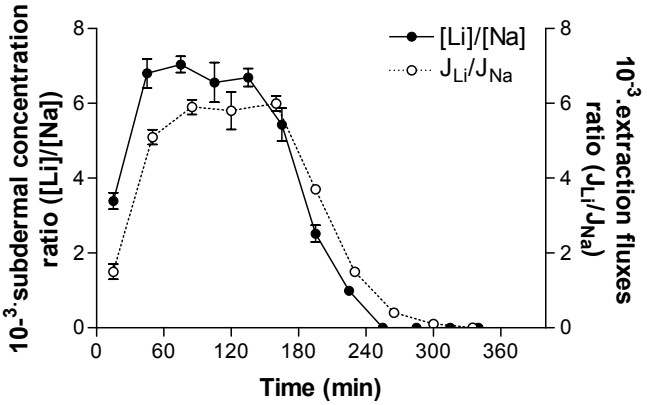

Figure 9: Simultaneous monitoring of lithium and sodium fluxes as a function of time. Data taken from [43].

pediatric, geriatric and chronically ill individuals are obvious examples. Therapeutic drug monitoring is similarly accessible via reverse iontophoresis and it is to be hoped that the size of the commercial markets here (relative to that for glucose monitoring) do not deter the ultimate realization of practical devices. The future holds promise, in particular, if analytical tools continue to evolve in term of sensitivity, specificity, and miniaturization, as they have in the recent past. If this is the case, then the application of reverse iontophoresis in "smart", feedback drug delivery systems, and in remote sensing, can be foreseen.

Aknowledgements

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Chapitre 2

Le monitoring non-invasif de la phénytoïne par ionophorèse inversée

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Résumé

Buts : Cette étude évalue, *in vitro*, la capacité de l'ionophorèse inversée à constituer une méthode non-invasive et alternative aux prises de sang pour le monitoring thérapeutique des médicaments fortement liés aux protéines plasmatiques. Ce travail démontre pour la phénytoïne, prise comme modèle, la relation de dépendance de l'extraction ionophorètique vis-à-vis des concentrations de phénytoïne (a) totale et (b) libre. Cette étude examine aussi l'aptitude de la technique (c) à suivre les effets d'une modification de la fixation protéique et (d) à devenir complètement non-invasive dès lors qu'un composé est utilisé comme standard interne pour sa calibration.

Méthodes : Les expériences d'extraction ionophorètiques ont été conduites *in vitro* en utilisant une peau dermatomisée d'oreille de porc. La solution subdermique consiste en un tampon physiologique contenant des concentrations thérapeutiques de phénytoïne, d'un standard interne, de sérum albumine humaine et/ou d'acide valproique.

Résultats : La forme ionisée de la phénytoïne fut extraite à l'anode par électromigration, tandis que la forme neutre fut extraite à la cathode par électroosmose. Une corrélation satisfaisante entre les quantités extraites de phénytoïne et les concentrations subdermiques fut observée. Il a été montré que l'extraction ionophorètique ne concernait que la fraction libre des concentrations subdermiques de phénytoïne et permettait de suivre les perturbations de l'équilibre de fixation protéique. L'acide acétique, introduit à concentration fixe dans le compartiment subdermique pour servir de « standard interne » fût extrait à l'anode. Il a été montré que le rapport des quantités extraites était proportionnel au rapport des concentrations subdermiques.

Conclusions : Ces résultats démontrent que la surveillance thérapeutique de la phénytoïne par ionophorèse inversée est possible dans son principe. Cette technique est sensible aux changements de concentration du médicament sous la peau et fournit un accès spécifique à la fraction libre de phénytoïne. De plus, le contrôle simultané d'un second analyte (standard interne) dont l'extraction reste indépendante de celle de la phénytoïne, permet une calibration de la méthode. Cette approche rend la technique complètement non-invasive. Si pour le moment, la lente stabilisation de l'extraction ionophorètique exclue la possibilité de suivre un profil pharmacocinétique complet, cette limitation n'altère pas les bénéfices non-invasifs de la méthode pour proposer une valeur moyenne suffisante au contrôle périodique des taux de phénytoïne chez les patients.

Mots clés : Ionophorèse, Transdermique, Monitoring thérapeutique, Phénytoïne

Non-invasive monitoring of phenytoin by reverse iontophoresis

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Abstract

Transdermal iontophoresis offers a non-invasive sampling method for therapeutic drug monitoring. This study examined whether iontophoretic extraction (a) is concentration dependent, (b) reflects the subdermal level of *unbound* drug, (c) follows protein binding changes, and (d) becomes truly noninvasive when a co-extracted compound is used as an internal standard for calibration. Iontophoresis was conducted *in vitro* using dermatomed pig-ear skin. The subdermal solution was a buffer containing phenytoin at therapeutic concentrations, an internal standard at fixed level, human albumin and/or valproic acid. The ionized form of phenytoin was recovered at the anode by electromigration, while the neutral form was extracted to the cathode by electroosmosis. A satisfactory correlation between the reverse iontophoretic extracted amount of phenytoin and the subdermal concentration was observed. Iontophoresis extracted only the free fraction of phenytoin. At steady state, reverse iontophoresis monitored changes in free drug concentration provoked in the subdermal compartment. Acetate was introduced at a fixed concentration into the subdermal compartment to act as an "internal standard". Subsequently, acetate and the ionized form of phenytoin were co-extracted to the anode. The ratio of the extracted amounts was proportional to the subdermal concentration ratio demonstrating a means by which the method may become truly non-invasive.

Keywords: Iontophoresis, Transdermal, Drug Monitoring, Phenytoin

I. Introduction

Phenytoin is a drug of choice in the control of grand mal epilepsy (1). The therapeutic window of phenytoin, however, is narrow and demands particular care with respect to dose titration to ensure efficacy and to avoid undesirable side-effects (2). Consequently, therapeutic monitoring for phenytoin is necessary, particularly in children and in the elderly for whom wide inter-individual differences are seen (3;4). As is not unusual for drugs with a narrow therapeutic index, phenytoin is highly protein-bound in the plasma, with only the limited free fraction (~10%) able to distribute across biological barriers (5). Effective monitoring, therefore, must report on the unbound drug in the plasma (6;7).

At present, the monitoring of phenytoin is performed via periodic blood sampling. Alternative, less invasive, approaches (for example, the measurement of drug levels in the sweat (8) or saliva (9;10)) have been investigated but have proven insufficiently reliable to be accepted in clinical practice (11). Clearly, a non-invasive method for sample collection, avoiding blood sampling, would be advantageous: the risk of infection for patient and health-care provider would be drastically reduced, and patient compliance would improve significantly making more frequent monitoring possible, even in an ambulatory setting. One might even envisage, in the long-term, a self-monitoring device that would provide a patient with an alert should the detected drug level trend towards certain pre-set limits.

Reverse iontophoresis is a maturing technology that has the potential to offer these benefits. The method involves the application of a low current (<0.5 mA/cm^2) to the skin so as to increase the percutaneous passage of ions and other compounds. Two mechanisms are implicated in iontophoresis: (i) electromigration, the direct interaction between the applied field and a charged ion, and (ii) electroosmosis, a convective solvent flow in the direction of anode to cathode, which results from the fact that the skin is net negatively-charged at physiological pH. Thus, cations and neutral, polar species are extracted at the cathode by reverse iontophoresis, while anionic species can be sampled at the anode. The method has been shown capable of extracting a diverse range of compounds including various electrolytes and drugs (12-14), glucose (15), phenylalanine (16) and urea (17). A commercial product for noninvasive glucose monitoring (the GlucoWatch Biographer®, Cygnus, Redwood City, CA), which is based on this technology, was approved by the U.S. Food & Drug Administration in 2001.

A clear limitation of reverse iontophoresis, however, is the need to acquire a blood sample for calibration purposes. That is, the concentration of the analyte of interest in the electrode collection chamber is not identical to that in the blood, and can be significantly smaller. The dilution factor is difficult to predict because it depends not only on the known volume of the "acceptor" compartment, but also upon either the transport number of the analyte (i.e., the fraction of the total charge carried across the skin by an ion of interest) or the charge on the skin itself (which determines quantitatively the electroosmotic flow); neither of these parameters are routinely available meaning that the most direct solution to the problem is a blood sample with which to calibrate the efficiency of reverse iontophoretic extraction. This is exactly the approach used with the GlucoWatch®, for example.

Recently, though, the use of a so-called "internal standard" calibration procedure has been proposed (13). The idea is that, in addition to the target analyte (A), a second compound (the "internal standard", IS), which is nominally present at a fixed concentration subdermally, is also extracted and quantified. The ratio of the extraction fluxes (J_A/J_{IS})

should reflect the ratio of their subdermal concentrations:

$$J_A/J_{IS} = K \,.\, [A]/[IS] \qquad \text{equation 1}$$

If the proportionality constant (K) and [IS] are indeed constant, then the extraction flux ratio gives direct access to the subdermal concentration of A:

$$[A] = (K^{\#})^{-1}.\, J_A/J_{IS} \qquad \text{equation 2}$$

where $K^{\#} = K/[IS]$. Here, acetate has been used as an internal standard with which to calibrate the anodal extraction of negatively-charged phenytoin (estimated to represent approximately 11% of the total amount present at pH 7.4). It is emphasized that acetate is used in this work as a model compound, in that its physiological concentration *in vivo* does vary over time. The unionized fraction of phenytoin was extracted to the cathode by electroosmosis; drug could therefore be quantified at both electrodes.

The *in vitro* investigation comprised three specific aims: (i) to verify the linear dependence of phenytoin extraction on the subdermal concentration of the drug, (ii) to demonstrate that reverse iontophoresis reports on free drug levels in the subdermal compartment, and (iii) to validate the "internal standard" calibration concept.

II. Methods

A. Materials

[4-^{14}C]-5,5-diphenylhydantoin (phenytoin) and [^{3}H]-acetate, sodium salt, were obtained from NEN™ Life Sciences Products, Inc. (Paris, France). Albumin (human fraction V), 5,5-diphenylhydantoin (phenytoin), sodium salt, sodium acetate, sodium valproate, Tris, Tris HCl, Mops and NaCl were all purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Deionized water (resistivity ≥ 18.2 MΩ.cm) was used to prepare all solutions.

Porcine ears were obtained fresh from the local abattoir (Annecy, France) and were cleaned under cold running water. The whole skin was excised from the outer region of the ear, removing carefully any underlying tissue or cartilage. Typically, the full thickness membrane was dermatomed to about 750 µm before being cut into smaller squares, wrapped in parafilm® and stored at –20°C until used (a period no longer than two months, but usually much shorter). All transport experiments employed skin from different "donors" (n≥3).

B. Iontophoresis

(a) Apparatus: *In vitro* experiments used either vertical diffusion cells (skin transport

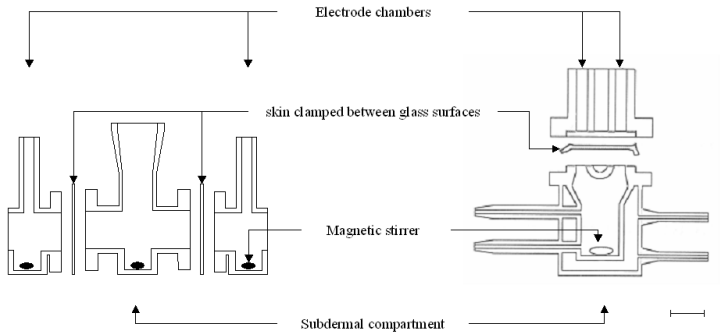

figure 1: Iontophoretic diffusion cells. The side-by-side, three compartment cell (left side), has an effective skin surface area of 1 cm^2; the subdermal volume is 3.5 mL, those of the electrode chambers are 1.5 mL. The vertical cell (right side) (18) has an effective area of 0.8 cm^2, a subdermal volume of 6.5 mL, and the electrode chambers volume is 1 mL. Scale bar is 1 cm.

area = 0.78 cm^2) (18), or a "side-by-side", three-compartment cell (effective area = $1cm^2$) (figure 1). It was found that the "side-by-side" cells allowed easier and more rapid exchanges of the electrode chamber and subdermal compartment solutions. Iontophoresis at constant current was enabled via the connection of Ag/AgCl electrodes to an appropriate power supply (KEPCO 1000M, Flushing, NY) (19). All iontophoresis experiments were performed in at least triplicate.

(b) Phenytoin extraction as a function of concentration: Full-thickness skin was clamped in vertical diffusion cells and was equilibrated for 30 minutes in the presence of the following background, pH 7.4-buffered, electrolyte solution: anode - Tris/Tris HCl (90 mM Tris), cathode - 32 mM Tris + 34 mM Mops, subdermal compartment - 32 mM Tris + 34 mM Mops + 133 mM NaCl. Then, the anode and cathode solutions were refreshed, while the subdermal chamber was replaced with the same buffer to which (a) phenytoin was added at one of the following concentrations: 4, 5, 8, 10, 18 and 26 µM, and (b) acetic acid was introduced at 50 µM. To facilitate analysis of the subsequent extraction samples, the subdermal solution was "spiked" with 0.02 µCi/mL of ^{3}H-acetate and 0.3 µCi/mL of ^{14}C-phenytoin. At pH 7.4, phenytoin, which has a pK_a of 8.3 (20), was calculated to be about 11% ionized. Iontophoresis was performed in two steps: for 6 hours at 0.4 mA, followed by 18 hours at 0.1 mA. At 3, 4, 5 and 6 hours post-initiation of iontophoresis, the current was stopped and the anode and cathode solutions were removed for analysis (of phenytoin and acetate) and replaced with fresh buffer. Equally, at the end of the 18-hour low-current step, anode and cathode solutions were taken for analysis. As a control, a passive diffusion experiment was also performed using the highest concentration of phenytoin. The electrode chambers, in this case, were sampled at 6 and 24 hours.

(c) Extraction of free, unbound phenytoin: Dermatomed skin was clamped in "side-by-side", three-compartment diffusion cells and allowed to equilibrate as before for 30 minutes. The electrode chamber solutions were then refreshed and the subdermal (central) compartment was filled with the same buffer to which human serum albumin (HSA) and phenytoin had been added in amounts that provided a known concentration of free, unbound drug. Low-current iontophoresis (0.1 mA) was then passed for 15h, at the end of which the electrode solutions were removed and replaced with fresh buffer. The current was then increased to 0.4 mA and drug extraction was determined by sampling the electrode chambers at 16, 17 and 18 hours post-initiation of iontophoresis. At 18 hours, in addition to replacing the electrode solutions with fresh buffer, the subdermal compartment was replaced with a new solution in which the free fraction of drug had been approximately doubled or halved. Current (0.4 mA) was re-started for a further 4 hours and hourly sampling of the electrode solutions was again performed. The phenytoin unbound concentration was altered in one of three ways by: (a) maintaining the drug level fixed at 80 µM and changing the HSA concentration from 22 to 44 $g.L^{-1}$ or vice versa; (b) changing the drug concentration from 80 µM to 40 µM or vice versa, while maintaining the level of HSA at 44 $g.L^{-1}$; and (c) addition of 542 µM valproate, which competes with phenytoin binding to albumin, while maintaining phenytoin and HSA concentrations at 80 µM and 44 $g.L^{-1}$, respectively. In all experiments, the subdermal solutions also contained 50 µM acetate ("spiked" with 0.02 µCi/mL of ^{3}H-Acetate) and ^{14}C-phenytoin (0.45 µCi/mL).

C. Determination of unbound phenytoin

Equilibrium dialysis experiments ($n \geq 3$) were performed using regenerated cellulose

membranes (Spectra/Por® 6, molecular weight cut-off 2000, Spectrum® Laboratories, Inc., Rancho Dominguez, California, USA). The latter were conditioned in pH 7.4 buffer (32 mM Tris + 34 mM Mops + 133 mM NaCl) for 30 minutes and then clamped between the two halves of a conventional "side-by-side" diffusion cell (area = 1 cm^2, volumes of "binding" solution and "dialyzed" solutions = 3.5 mL and 1.5 mL, respectively). The two compartments were filled with fresh buffer and the cell was allowed to equilibrate for a further 30 minutes. Subsequently, the cell was emptied and then refilled with 1.5 mL of buffer in the "dialyzed" compartment and 3.5 mL of the phenytoin/albumin or phenytoin/albumin/ valproate test solution in the "binding" compartment. The two compartments were magnetically stirred for 4 days at room temperature at the end of which 100 µL of each compartment was removed for analysis, and the extent of protein binding was calculated.

D. Analysis

All samples were mixed with 5 mL of scintillation cocktail (Ultima Gold XR, Packard Instruments SA, Rungis, France), and then analysed by for ^{14}C-phenytoin and ^{3}H-acetate by liquid scintillation counting (LS 6500, Beckman Instruments France SA, Gagny, France).

E. Statistics

Data analysis and linear regression were performed using Graph Pad Prism V.3.02. (Graph Pad Software, Inc. San Diego, California, USA). Linear regressions were always followed by the corresponding ANOVA. All the regressions reported in this work were significant (P<0.001). Paired t-tests were carried out with SigmaStat™ for Windows V.2.03 (SPSS Science Software Gmb, Erkrath, Germany); the significance level was fixed at P<0.01.

III. Results and Discussion

The passive extraction fluxes of phenytoin after 6 and 24 hours of diffusion were 0.1 ± 0.1 $pmol.cm^{-2}.h^{-1}$ and 1.2 ± 0.9 $pmol.cm^{-2}.h^{-1}$, respectively, and were negligible in comparison to the iontophoretic values.

The first objective of this study was to demonstrate proportionality between the reverse iontophoretic extraction flux of phenytoin and its subdermal concentration. Figure 2 demonstrates that this was indeed the case. Over a therapeutic range of subdermal levels, phenytoin extraction rates increased linearly. Extraction to both the anode and the cathode chambers was observed. Coincidentally, the electromigration transport of negatively-charged phenytoin (representing about 11% of that present at pH 7.4) to the anode was quantitatively quite similar to the

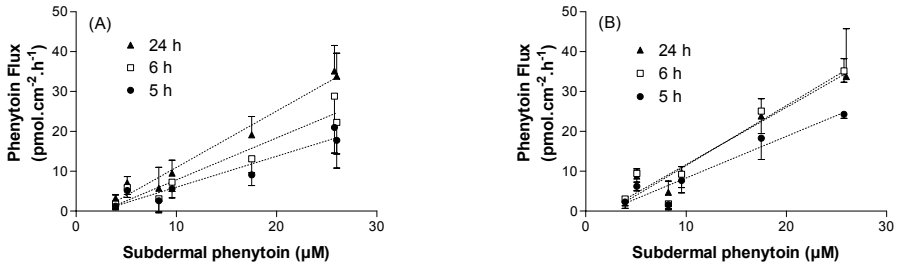

figure 2: Iontophoretic extraction fluxes of phenytoin as a function of time and subdermal concentration (A) at the anode, and (B) at the cathode. Each data point represents the mean ± standard deviation (n=3). Lines of linear regression are drawn through the data.

table 1: Phenytoin extraction flux (J) is proportional to the subdermal drug concentration (C) ($J = \beta.C + intercept$[a]) at various times post-initiation of reverse iontophoresis.

Electrode	Time (h)	Current (mA)	β[b] (µm/h)	r^2
Anode	3	0.4	3 ± 1	0.63
	4	0.4	5 ± 1	0.72
	5	0.4	8 ± 1	0.75
	6	0.4	11 ± 1	0.79
	24	0.1	14 ± 1	0.90
Cathode	3	0.4	4 ± 1	0.76
	4	0.4	7 ± 1	0.80
	5	0.4	11 ± 1	0.85
	6	0.4	15 ± 2	0.86
	24	0.1	14 ± 1	0.87

[a]The absolute value of the intercepts was 3.6 $pmol.cm^{-2}.h^{-1}$ or less
[b]mean ± SD (n=3)

electroosmotic transport of the uncharged drug (~89% of the total) to the cathode. It is worth noting that after 6 hours of reverse iontophoretic extraction, the amounts of phenytoin recovered at the skin surface were on the order of 300 times greater than the passive values.

During the first 6 hours of the extraction experiment, the current was 0.4 mA. From figure 2, it is apparent that the efficiency of extraction was greater at 6 hours than at 5 hours, suggesting that steady-state transport had not been attained. This conclusion is reinforced by the data at 24 hours, following iontophoresis at 0.1 mA for an 18-hour period: despite the lower current, during this time period, the extraction fluxes closely matched those seen at 6 hours. The linearity of the extraction process as a fraction of subdermal phenytoin concentration is summarized in table 1.

The precise reason why the time to steady-state is prolonged has not been unequivocally elucidated. Certainly, the lipophilicity of the drug plays a role. For example, like phenytoin, valproate requires several hours of constant current to achieve steady-sate extraction (13). On the other hand, a small cation, like lithium, reaches steady-state within an hour or less (14). Clearly, there are also endogenous ions within the skin at the moment that current passage is initiated. Due to their presence *in situ*, these species will of necessity contribute to carrying the charge at the beginning of an iontophoresis procedure.

The efficiency and linearity of electroosmosis extraction (with respect to the subdermal drug concentration) are also demonstrated in figure 2 and table 1. The flux measured at 0.1 mA corresponds to a convective solvent flow of 1.6 ± 0.1 $\mu L.cm^{-2}.h^{-1}$, a value in good agreement with that previously reported in the literature (2-6 $\mu L.cm^{-2}.h^{-1}$ at 0.2 mA) (21). Again, the

table 2: Free fraction of phenytoin (α) determined by equilibrium dialysis.

Phenytoin (µM)	Albumin ($g.L^{-1}$)	Valproate (µM)	α[a]	Free phenytoin (µM)	n[b]
80	44	-	0.11 ± 0.011	8.8	18
40	44	-	0.11 ± 0.004	4.4	3
80	22	-	0.21 ± 0.007	16.8	6
80	44	542	0.19 ± 0.012	15.2	6

[a]mean ± SD
[b]n=number of replicates

approach to steady-state is rather slow, and similar to that seen for electromigration towards the anode.

From a practical standpoint, the need for steady-state is not necessarily essential. If subdermal levels are changing rapidly, then a long extraction period makes little sense. However, in the case of drugs like phenytoin (or valproate, or lithium), therapeutic monitoring is useful for checking and/or re-setting steady-state plasma levels. The situation is not like glucose monitoring, for example, where frequent measurements must be made so that the sometimes rapid increases and decreases in blood sugar can be observed and acted upon as quickly as possible.

The second goal of the investigation was to demonstrate that reverse iontophoresis extraction reflects the subdermal level of free drug. To prove this hypothesis, experiments were performed in which the unbound concentration of phenytoin was deliberately changed at a specific time. The reverse iontophoretic extraction before and after this change was then monitored. The free fraction of drugs was altered by (i) changing protein concentration while keeping the drug level constant, (ii) changing the drug concentration at fixed albumin level, and (iii) adding valproate, which competes with phenytoin for protein binding sites.

The results of the equilibrium dialysis experiments, which were used to determine the unbound drug levels, are in table 2. It is first noted that the free fractions observed (~11%) are consistent with those reported in the literature for the "normal" range of phenytoin plasma concentrations (40-80 µM) (22-24). Second, as expected, a decrease in protein by a fraction of two induced a doubling of the amount of unbound drug (25). Third, the introduction of valproate led to a more than 70% increase in free phenytoin; data in the literature support a similar phenomenon (with increases from 30% to 100% having been observed) (22).

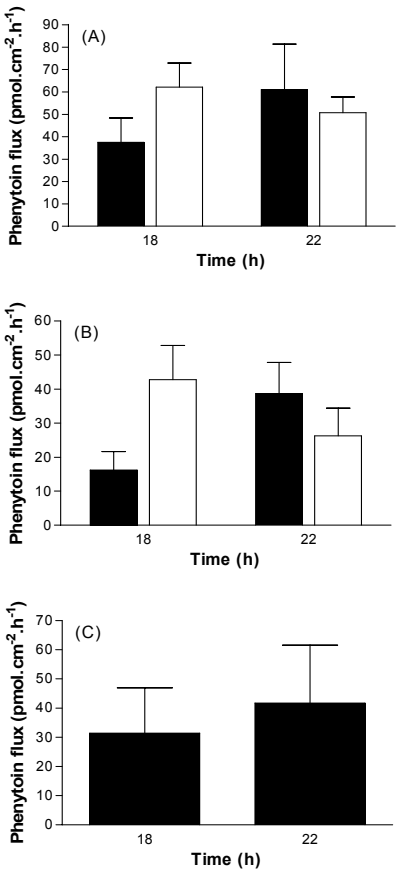

figure 3: Sum of anodal and cathodal reverse iontophoretic extraction fluxes of phenytoin (mean ± SD; n≥6) in response to changes in the free fraction of drug in the subdermal chamber. (A) Total [phenytoin] fixed at 80 µM; [albumin] changed from 44 to 22 $g.L^{-1}$ (solid bars) or from 22 to 44 $g.L^{-1}$ (open bars). (B) [albumin] fixed at 44 $g.L^{-1}$; total [phenytoin] changed from 40 to 80 µM (solid bars) or from 80 to 40 µM (open bars). (C) Total [phenytoin] and [albumin] fixed at 80 µM and 44 $g.L^{-1}$; [valproate] changed from 0 to 542 µM. Changes described were made immediately after measurements taken at t=18 hours. The corresponding free fractions of phenytoin in these experiments are in table 2.

The reverse iontophoretic extraction fluxes of phenytoin were sensitive to these specifically induced changes in the free fraction of the drug (P<0.01, figure 3). The responses were essentially identical at the anode and cathode and figure 3 plots, therefore, the sum of the extraction fluxes of the charged and unionized drug. In these experiments, the extraction was monitored at 16, 17 and 18 hours post-initiation of iontophoresis and the flux was shown to be constant. The changes were then provoked and the extraction rate of the drug was followed hourly for a further 4 hours. Figure 3A and 3B show that reverse iontophoresis detected correctly the increase and decrease in free phenytoin when either albumin concentration was decreased or increased, respectively, at fixed drug level, or when phenytoin concentration was increased or decreased at fixed protein level. Figure 3C shows that competitive protein binding by valproate (mimicking a not untypical situation in clinical practice) also elicited, as anticipated, a higher free fraction of phenytoin and a greater extraction flux.

The third aim of this research was to examine whether the reverse iontophoretic sampling procedure may be performed without the need for a calibration blood sample. With this in mind, a second compound (acetate) at fixed concentration (50 µM) was introduced into the subdermal compartment with phenytoin, whose concentration was varied over a therapeutic range. The idea is that the measured extraction flux ratio (phenytoin/acetate) at the anode should be proportional to the corresponding ratio of their subdermal concentrations (equation 1). If the proportionality constant is indeed constant then, giving that the subdermal acetate level is fixed, measurement of the extraction flux ratio allows direct access to the "systemic" phenytoin concentration (equation 2).

It was first necessary to show that the extraction flux of acetate remained constant while the subdermal concentration of phenytoin was allowed to vary. This was indeed the case as is illustrated in figure 4. As anticipated for the smaller, more water-soluble acetate ion, its extraction flux reached a steady-state flux rapidly (within 3 hours – see figure 5) and, when the current in this experiment was reduced from 0.4 to 0.1 mA

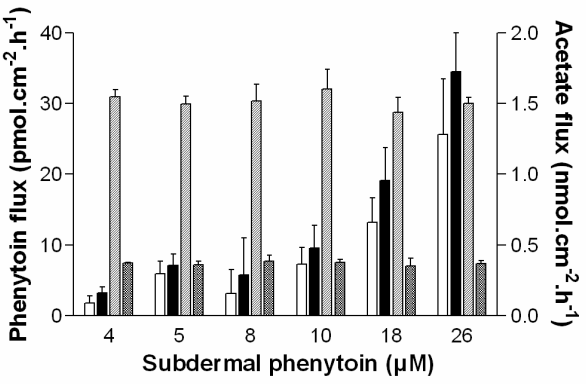

figure 4: Simultaneous anodal extraction fluxes of phenytoin and acetate as a function of the subdermal phenytoin concentration; the subdermal concentration of acetate was fixed at 50 µM. Reverse iontophoretic extraction at 6 hours (current 0.4 mA) and at 24 hours (current 0.1 mA) is shown: □ and ▨ are the phenytoin and acetate fluxes, respectively, at 6 hours; ■ and ▩ are the corresponding values at 24 hours. Each bar indicates the mean ± SD for at least 3 replicates experiments.

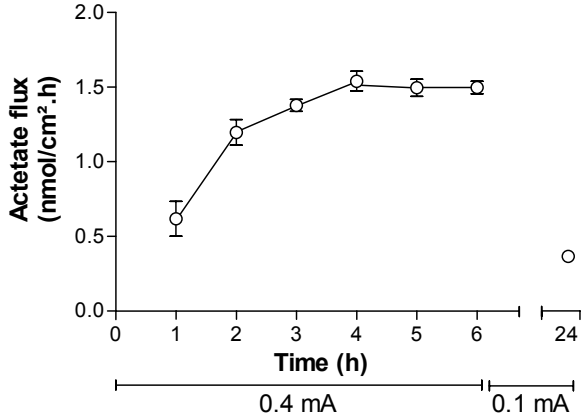

figure 5: Anodal, reverse iontophoretic extraction of acetate as a function of time and applied current. Each data point is the mean ± SD of 6 replicates. The error on the value at 24 hours is smaller than the size of symbol and cannot be visualized.

for the period from 6 to 24 hours, its transport fell by a factor of 4.

The test of the "internal standard" hypothesis (equation 2) is presented in figure 6. The values of $K^{\#}$, calculated using the extraction fluxes measured at 5, 6 and 24 hours were 0.5 ± 0.1, 0.7 ± 0.1 and 3.9 ± 0.3 mM^{-1}, respectively (r^2 values for the corresponding regressions were 0.76, 0.79 and 0.90). The fact that $K^{\#}$ is not constant with time reflects the fact (as has already been pointed out) that phenytoin requires a considerably longer time to reach steady-state extraction than acetate. However, this does not necessarily invalidate the idea behind the "internal standard" hypothesis with respect to its practical application. Provided a specific time of extraction is selected, then the value of $K^{\#}$ operative at that moment should then permit an estimate of the subdermal phenytoin concentration to be made.

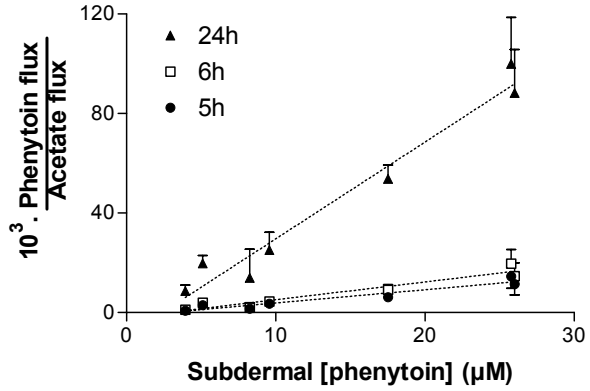

figure 6: The ratio of the anodal phenytoin and acetate fluxes plotted as a function of subdermal phenytoin concentration after 5, 6 and 24 hours of iontophoresis. The results conform well to equation 2, providing support for the "internal standard" concept. Each point represents the mean ± SD of at least 3 replicates.

IV. Conclusion

In conclusion, the results presented in this paper demonstrate, in principle, that transdermal therapeutic monitoring of phenytoin by reverse iontophoresis is possible. The sampling procedure is sensitive to changes in the drug's concentration beneath the skin and provides access, specifically, to the free fraction of phenytoin that is not protein-bound. Furthermore, the simultaneous sampling of a second analyte, whose concentration remains constant relative to the variation in phenytoin levels, permits an "internal standard" calibration such that the approach is rendered completely non-invasive. However, the lipophilicity of phenytoin is such that its reverse iontophoretic extraction attains a steady-state rate only slowly. While this may not detract from the usefulness of the method to provide a simple means to monitor drug levels periodically in patients receiving chronic phenytoin therapy, this limitation does, for the moment, exclude the possibility of tracking a complete pharmacokinetic profile subsequent (for example) to a single oral dose.

Abbreviations

J: Extraction flux (units: $pmol.cm^{-2}.h^{-1}$)

K: Proportionality constant (units: µm/h) between extraction flux ($J_{phenytoin}$) and subdermal phenytoin concentration.

$K^{\#}$: Proportionality constant (units: mM^{-1}) between ratio of extraction fluxes ($J_{phenytoin}/J_{acetate}$) and subdermal phenytoin concentration.

Acknowledgments

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Chapitre 3

L'ionophorèse inversée comme outil non-invasif pour le monitoring du lithium et les études pharmacocinétiques

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Résumé

Buts : Cette étude évalue le potentiel de l'ionophorèse inversée à être proposée comme un outil alternatif et non-invasif dans la surveillance thérapeutique des médicaments et dans la réalisation des études pharmacocinétiques. Le lithium, nécessitant une surveillance et un contrôle fréquent fut choisi comme molécule modèle. Les objectifs étaient de (a) démontrer la relation linéaire existant entre l'extraction ionophorètique et les concentrations subdermiques, (b) d'examiner la capacité de l'ionophorèse à suivre des variations brusques de concentration subdermique, (c) d'évaluer le potentiel de l'ionophorèse comme outil dans les études de pharmacocinétique et (d) d'examiner la validité de la calibration de la méthode par un standard interne dans le but de rendre cette méthode complètement non-invasive.

Méthodes : L'extraction transdermique a été menée *in vitro* en utilisant de la peau dermatomisée d'oreille de porc. La solution subdermique consistait en un tampon physiologique contenant des concentrations de lithium en rapport avec sa marge thérapeutique ainsi que deux standards internes : le sodium et le potassium à concentrations physiologiques fixes. La concentration de lithium dans le compartiment subdermique a été modifiée de deux manières : soit brusquement, soit en simulant des profils monocompartimentaux.

Résultats : Le lithium a été extrait à la cathode par électromigration. Une bonne corrélation entre les concentrations subdermiques et les quantités extraites a été observée. L'ionophorèse inversée a démontré sa capacité à suivre des modifications brusques de concentrations subdermiques et des profils pharmacocinétiques. Il a été montré que les paramètres pharmacocinétiques (comme la constante d'élimination) peuvent être directement, et de manière non-invasive, estimés par cette méthode de monitoring.

Conclusions : Cette étude confirme le potentiel de l'ionophorèse inversée à proposer une méthode alternative aux prélèvements sanguins pour le monitoring thérapeutique du lithium et la réalisation d'études pharmacocinétiques de manière complètement non-invasive.

Mots clés : Ionophorèse, Ionophorèse inversée, Monitoring thérapeutique, Lithium, Extraction transdermique

Reverse iontophoresis as a non-invasive tool for lithium monitoring and pharmacokinetic profiling

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Abstract

Purpose: Transdermal iontophoresis was investigated as a non-invasive tool for drug monitoring and pharmacokinetic profiling. Lithium, a frequently monitored drug, was used as a model. The objectives were: (a) to demonstrate the linear dependence of the iontophoretic extraction flux of lithium on the subdermal concentration of the drug, (b) to evaluate the capacity of iontophoresis to monitor sudden changes in the subdermal level, (c) to investigate the utility of reverse iontophoresis as a tool in pharmacokinetic studies, and (d) to examine the validity of an internal standard calibration procedure to render the method completely non-invasive.

Methods: Transdermal, iontophoretic extraction was performed *in vitro* using dermatomed pig-ear skin. The subdermal solution consisted of a physiological buffer containing lithium chloride at concentrations in the therapeutic range and two putative internal standards, sodium and potassium, at fixed physiological levels. The subdermal concentration of lithium was changed either in a stepwise fashion or by simulating one of two pharmacokinetic profiles.

Results: Lithium was extracted via electromigration to the cathode. A excellent correlation between subdermal lithium concentration and iontophoretic extraction flux was observed. Iontophoresis tracked sudden concentration changes and followed kinetic profiles. In addition, the effective elimination rate constant could be directly, and non-invasively, estimated from the extraction flux data.

Conclusions: Reverse iontophoresis is a potentially useful and non-invasive tool for lithium monitoring.

Keywords: Iontophoresis, Reverse iontophoresis, Therapeutic drug monitoring, Lithium, Transdermal extraction

I. Introduction

Currently, therapeutic drug monitoring and pharmacokinetic studies depend principally upon the quantification of the molecule in one or more blood samples. The invasive nature of the procedure, the associated risks of infection, the need for trained personnel and, for some populations (e.g., neonates), the technical difficulty, limit the frequency and complexity of these studies. As a result, drug kinetics in certain patient groups are poorly understood and drug monitoring, in general, is performed much less often than it should.

There is a clear need, therefore, for the development of non-invasive techniques, which would be much better accepted by the patient and would offer the possibility of frequent and ambulatory self-monitoring (1, 2). Paediatric, neonatal, and geriatric patients, as well as the chronically and critically ill, would benefit most from the availability of such tools. For example, manic-depressive patients receiving chronic lithium therapy would profit from frequent monitoring that does not demand repeated visits to the hospital. These patients are carefully supervised because of (a) the wide inter-subject variability observed in dose requirement and tolerance to the drug, (b) the very narrow therapeutic index, and (c) a half-life, which depends on kidney function, sodium intake and age (3). Monitoring is initially performed to adjust the dose; daily, weekly and, finally, monthly evaluations are carried out during the first 6 months. Thereafter, lithium levels are checked at least every three months to detect drifts in concentration (4). The latter may be due to age, pregnancy, low salt diet, fever, infection, drug interactions, other medical problems, and/or poor compliance with the dosage regimen (4-6). At present, lithium monitoring requires blood sampling. Attempts to use either saliva or urine as alternative matrices have not been successful (3, 7-9).

Reverse, transdermal iontophoresis has been proposed as an alternative technique for non-invasive monitoring (10-15). Potential applications in clinical chemistry and therapeutic drug monitoring have been identified. Iontophoresis involves the application of a small electrical current (<0.5 mA/cm²) to the skin (16, 17), and results in enhanced transport across the membrane via two possible mechanisms (18-20). The first, electromigration, only concerns ions, which carry the current through the skin towards the electrode of opposite polarity. Thus, in reverse iontophoresis, anions are extracted at the anode and cations (such as lithium) at the cathode (negative electrode). The ion flux is related to the intensity of current applied via equation 1:

$$J_a = \frac{I.t_a}{Z_a.F} \qquad \text{Equation 1}$$

where, J_a, t_a and z_a are the flux, transport number and valence, respectively, of the ion "a"; I is the intensity of current applied, and F is Faraday's constant. It has been shown that, in the presence of competing ions, the transport number (i.e., the percentage of the charge carried) of a given ion is proportionally related to its concentration in the donor solution (21). Thus, in reverse iontophoresis, it is expected that the flux of a given analyte should be related to its concentration in the subdermal fluid. The second mechanism of transport is electroosmosis, which is a convective solvent flow, in the anode-to-cathode direction, due to the fact that the skin has a net negative charge. This flow increases the transdermal transport of neutral (e.g., glucose) and zwitterionic species and supplements the electromigration of cations. It has also been shown that the electroosmotic transport of an analyte is directly proportional to the concentration of the species present in the solvent (19, 22).

The Glucowatch Biographer® (Cygnus, Inc., California, USA), which monitors blood sugar, is the only approved reverse iontophoretic system on the market (23). Before use, the device has to be calibrated via

a conventional finger-stick so that the amount of electroosmotically-extracted glucose (measured *in situ* by the appparatus), in a defined period of current passage, can be related to the corresponding blood level. At present, it is unknown whether iontophoretic devices relying on electromigrative extraction will also require a similar calibration; such would be the case, for example, if the transport number of a specific ion demonstrated a significant inter-individual variability *in vivo*.

In any case, the development of a non-invasive calibration procedure can clearly be identified as a sensible priority for future applications of reverse iontophoresis in drug monitoring and, recently, the use of an "internal standard" approach has been proposed (10, 11, 24). The procedure takes advantage of the fact that iontophoretic extraction is not specific. For example, in the case of lithium, several other cations will be simultaneously extracted at the cathode. If one of these ions, which may be referred to as the "internal standard", is present in the body at a relatively constant concentration, then its iontophoretic flux ($J_{I.S.}$) would be expected to be constant as well. It follows that the ratio of the extraction fluxes, $J_{Li}/J_{I.S.}$ should be directly proportional to the ratio of their respective concentrations ($C_{Li}/C_{I.S.}$) (10, 11, 24). Given that $C_{I.S.}$ is constant, $J_{Li}/J_{I.S}$ becomes directly proportional to C_{Li}:

$$J_{Li}/J_{I.S.} = R_{I.S.} = \gamma^{\#}. C_{Li} \qquad \text{Equation 2}$$

where $\gamma^{\#}$ is a constant. This hypothesis is tested here using sodium and potassium as candidate internal standards for lithium.

In summary, this work explores the potential of reverse iontophoresis as a non-invasive procedure for lithium monitoring and for pharmacokinetic profiling. In particular, the following questions have been addressed: (i) Are iontophoretic extraction fluxes of lithium proportional to the corresponding subdermal concentrations? (ii) Is iontophoresis capable of following sudden changes in the lithium subdermal concentration? (iii) To what extent may reverse iontophoresis be useful for pharmacokinetic studies? (iv) Does the "internal standard" calibration approach work?

II. Materials and methods

A. Materials

8 M LiCl solution, NaCl, KCl, NaOH, KOH, Hepes, Tris, TrisHCl, Mops, Ag wire 99.9%, AgCl 99%, Pt 99.9% were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Deionized water (resistivity ≥ 18.2 MΩ.cm) was used to prepare all solutions.

B. Skin preparation

Porcine ears were obtained fresh from the local slaughterhouse (S.O.D.E.X.A., Annecy, France) 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. Both full thickness and dermatomed (~750 µm) skin were used. The skin was wrapped individually in Parafilm® and maintained at –20°C for no longer than two months. All experiments were performed with 3 to 6 replicates, using skin samples originating from different pigs.

C. Equipment

Two types of iontophoretic cells were used: (a) vertical iontophoretic cells (25) with an effective transport area of 0.78 cm², a 6.5 ml subdermal volume and 1 ml electrode chambers; and (b) side-by-side three-compartment cells (11) with a 1 cm^2 skin surface area, a 4.54 ± 0.15 ml subdermal compartment and 1.5 ml electrode chambers. Access ports to the subdermal compartment permitted the lithium concentration to be perfused at a fixed level, to be changed abruptly, and to be modulated continuously

over time so as to mimic a pharmacokinetic profile. A manual power supply (either a KEPCO 1000M, Flushing, NY, USA or a Yokogawa 7651, Tokyo, Japan) was used to deliver a constant current via Ag/AgCl electrodes (26).

D. Fixed-concentration extraction experiments

Full-thickness skin was clamped in vertical iontophoretic cells. First, the subdermal and anodal compartments were filled with a pH 7.4 buffer comprising 25 mM Hepes and 133 mM NaCl. The cathodal compartment was filled with a pH 7.4 buffer comprising 25 mM Hepes and 10 mM KCl.

After 30 minutes equilibration, the anodal and cathodal solutions were refreshed. The subdermal chamber was filled with the "donor" solution, which consisted of the same buffer to which lithium chloride was added at one of three different concentrations (0.6, 1.0 and 1.5 mM) corresponding to the drug's therapeutic range. Iontophoresis was performed for 5 hours by applying a constant current of 0.4 mA via Ag/AgCl electrodes. The current was stopped hourly to permit the collection and replacement of the entire electrode chambers solutions. Three replicates were made. The samples were assayed for lithium by graphite furnace atomic spectrometry (GFAS).

E. Stepwise concentration change experiments

Dermatomed skin was clamped between the three compartments of side-by-side cells. During a 30-minute equilibration period, the subdermal compartment was filled with 3.5 ml of a pH 7.4 buffer solution containing 32 mM Tris, 34 mM Mops and 133 mM NaCl. The anodal chamber was filled with a pH 7.4 buffer comprising 90 mM Tris/Tris HCl, while the cathodal chamber was also buffered at physiological pH with 32 mM Tris and 34 mM Mops. Subsequently, the anodal and cathodal solutions were refreshed. The subdermal chamber was filled with the same buffer to which 4 mM potassium chloride and lithium chloride at either 0.95 mM (first experiment) or 1.7 mM (second experiment) were added. The concentration of LiCl in the subdermal donor solution was then changed in a stepwise fashion at 120 and 210 minutes: in the first experiment, to 2.7 mM and 1.8 mM, respectively; in the second, to 0.6 and 1.1 mM.

Iontophoresis was performed for 5 hours at a constant current of 0.4 mA. Six replicates were carried out. Every 30 minutes, the current was stopped and the entire electrode chamber solutions were sampled and refilled with fresh buffer. The samples were assayed for lithium by graphite furnace atomic spectrometry (GFAS) and for potassium and sodium by flame atomic absorption spectrometry (FAAS).

F. Concentration-profile kinetic experiments

Dermatomed skin was clamped in side-by-side cells. After an equilibration period as described before, the solutions in the three compartments were refreshed. Then, the lithium content of the subdermal solution was varied to simulate the plasma concentration profile observed after either an IV bolus or a continuous infusion (see below). Iontophoresis was performed at a constant current of 0.4 mA for 5 hours. Every 30 minutes, the current was stopped (for a period of ~5 minutes) to permit the collection and the replacement of the entire electrode solutions. At the midpoint of each of iontophoretic period, 10 µl of the subdermal solution were sampled and the actual concentrations of lithium, sodium and potassium therein were quantified by ionic chromatography with conductimetric detection, allowing their iontophoretic extraction fluxes to be calculated.

Intravenous bolus: A syringe pump (Genie 8, Kent Scientific Corporation, Torrington CT, USA) infused the subdermal buffer (pH 7.4, 32 mM Tris, 34 mM Mops, 133 mM NaCl, 4 mM KCl) at a rate of 1 ml/h. After one hour of iontophoresis, 6.2 µl of a 0.8 M aqueous solution of LiCl were directly added via a bolus injection into the subdermal compartment of each cell.

Constant rate infusion: A syringe pump infused the subdermal buffer as before, but at a rate of 4 ml/h. After one hour of iontophoresis, the composition of the infused solution was modified to incorporate 1.4 mM LiCl.

G. Analytical techniques

Graphite furnace atomic absorption spectrometry with Zeeman effect quantified lithium at 670.8 nm (Perkin Elmer 4100 ZL, Norwalk, CT, USA). The samples were diluted 20-fold in 0.2% HNO_3 before injection.

Flame atomic absorption spectrometry (Perkin Elmer AA Analyst 300, Perkin-Elmer Corporation, Norwalk, CT, USA) was used to measure sodium and potassium concentrations in iontophoretic and donor samples. The ions were quantified at 589.1 nm and 769.9 nm, respectively, after a 10-fold dilution of the samples in 10% HNO_3.

Ionic chromatography was used to quantify lithium, sodium and potassium. The Dionex DX-600 system (Voisins le Bretonneux, France) was equipped with a GP-50 pump, and an AS-50 thermal compartment (25°C). A 6 mM H_2SO_4 mobile phase was pumped (1 ml/min) through a CS-16 cationic column. Detection involved a ED-50 detector and an Atlas suppressor (61 mA) .

H. Statistics

Data analysis, linear and non-linear regressions were performed with Graph Pad Prism V.4.0 (GraphPad Software Inc. San Diego, USA). All linear regressions shown in this work were significant ($p<0.001$). The data from each iontophoresis cell was individually fitted to the corresponding regression equation, and the "kinetic" values derived correspond to the average and standard deviation (SD) of 6 cells. Kruskal-Wallis and repeated measures ANOVA analysis were performed with SigmaStat V.2.03 (SPSS Science Software GmbH, Erkrath, Germany). The statistical significance level was fixed at $P<0.05$. $(AUC)_{0\rightarrow t}$ was determined via the trapezoidal method (Prism V.4.); $(AUC)_{t\rightarrow\infty}$ was calculated from the ratio of the last value measured to the elimination constant rate (27).

III. Results and discussion

A. Fixed-concentration extraction experiments

The objective of this first set of experiments was to verify the concentration dependence of the lithium iontophoretic extraction flux over the therapeutic range. Lithium, a positive ion, was extracted at the cathode (negative electrode) as expected. Figure 1 shows that lithium flux stabilized after approximately 2-3 hours of iontophoresis. This behaviour has been previously observed. The delay results from the fact that, when the current is started, the most readily available charge carriers are the endogenous ions already present inside the skin (18); only after this "reservoir" is depleted can lithium assume its full role in transporting charge across the barrier. Lithium transport became steady after a few hours of iontophoresis, in a similar manner to that reported for acetate, but more rapidly than has been observed for valproate (~5 hours) (10) and phenytoin (>10 hours) (11). The iontophoretic lithium flux, when the "donor" concentration was 0.6 mM, reached 20.0 ± 1.0 nmol.h^{-1} a value nearly an order of magnitude greater than that found for valproate (2.3 nmol.h^{-1}) under similar conditions (10). This illustrates the critical role of the

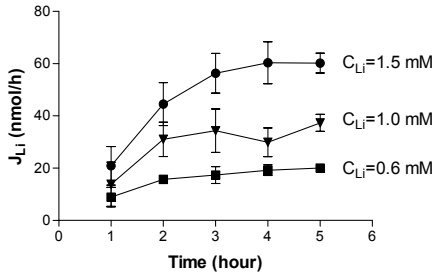

Figure 1: Reverse iontophoretic extraction fluxes of lithium as a function of time and subdermal lithium concentration. Each data point represents the mean ± standard deviation (n=3).

physicochemical properties (molecular weight, mobility and polarity) of the analyte of interest in determining the feasibility of iontophoretic extraction. Lithium, being smaller, more mobile and much less lipophilic than valproate, competes much better in carrying the charge across the skin. Furthermore, electroosmosis assists lithium transport while reducing that of the anionic valproate (19).

The relationship between lithium flux and subdermal concentration was analyzed by linear regression (Equation 3), and the results are in Table I:

$$J_{Li} = \gamma . C_{Li} \qquad \text{Equation 3}$$

The values of the slope (γ) progressively increased until the third hour of extraction. Satisfactory correlation coefficients were observed as early as 2 hours after the initiation of iontophoresis (r^2 = 0.85), with the best correlation obtained at 5 hours (r^2 = 0.97). Overall, this first experiment demonstrated that a linear relation between lithium extraction flux and the subdermal concentration of the drug is established after a relatively short sampling time.

B. Stepwise concentration change experiments

The objective here was to investigate whether the iontophoretic extraction procedure could follow abrupt subdermal concentration changes. The experiments began with an initial 2-hour period of current passage during which the lithium subdermal concentration was maintained constant at either 0.95 mM or 1.7 mM (Figures 2 (a) and (b), respectively). During this time, the iontophoretic fluxes increased and reached steady values faster than observed before. This was probably due to the smaller thickness of the skin membrane used

Table I: Linear regressions of the iontophoretic extraction fluxes of lithium (J_{Li} in units of nmol/h) as a function of the drug's subdermal concentration (C_{Li} in mM) after different times of iontophoresis (data in Figure 1), according to the equation: $J_{Li} = \gamma \cdot C_{Li}$ + Intercept[a].

Time (h)	γ (µl/h)	r^2
1	13 ± 6	0.43
2	31 ± 5	0.85
3	42 ± 6	0.89
4	44 ± 7	0.87
5	43 ± 3	0.97

[a] The absolute values of the intercepts were 9 nmol/h or less

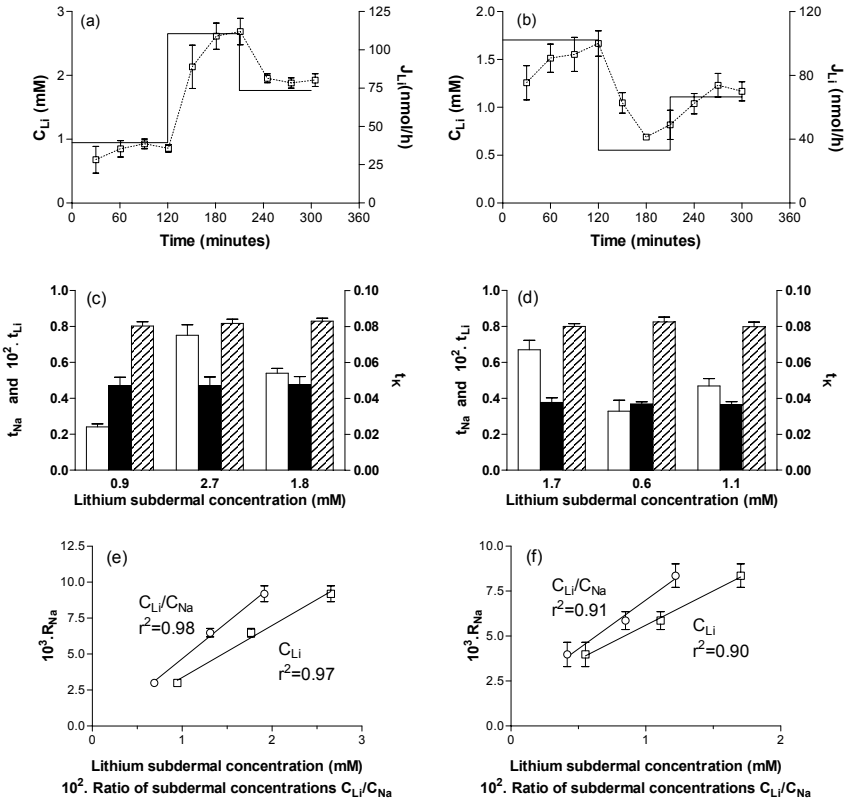

Figure 2: Monitoring of lithium subdermal concentration changes: (a) first experiment, (b) second experiment. The continuous lines indicate the stepwise changes in the subdermal lithium concentrations. The open squares are the lithium iontophoretic fluxes. Each data point represents the mean ± standard deviation (n=6). Transport numbers of lithium (t_{Li}, open bars) potassium (t_K, solid bars) and sodium (t_{Na}, hatched bars) determined from the iontophoretic fluxes in (c) the first experiment, and (d) the second experiment. Linear regressions between the ratio of extraction fluxes (R_{Na}) and either the lithium subdermal concentration or the ratio of subdermal concentrations (C_{Li}/C_{Na}) determined using the data from (e) the first experiment, and (f) the second experiment.

(dermatomed *versus* full-thickness tissue) and the shorter sampling intervals employed (30 minutes *versus* 1 hour), which allowed the kinetics to be determined more precisely.

The concentration of LiCl in the subdermal donor solution was then changed in a stepwise fashion at 120 and 210 minutes: in the first experiment, to 2.7 mM and 1.8 mM, respectively (Figure 2 (a)); in the second, to 0.6 and 1.1 mM (Figure 2 (b)). These sudden changes, which are much more abrupt than any possible in an *in vivo* situation of course, were carried out to test the responsiveness of reverse iontophoresis to such variations.

Figure 2 shows that iontophoresis was quite efficient in following the stepwise changes in the subdermal composition. Lithium fluxes responded appropriately to the new conditions established in each case and reached new steady values after a relatively

short delay. As the "physiological" buffer used remained constant, increasing or decreasing the lithium concentration conferred, respectively, a better or worse chance for the drug to compete to carry the charge across the skin (i.e. to adopt a higher or smaller transport number (18)).

Taken together, the first two sets of experiments confirm that reverse iontophoresis of a highly mobile drug, such as lithium, can provide credible information about concentration changes occurring in the internal medium with a relatively short time-lag. It should be emphasized that such delays seen under *in vitro* conditions may not be observed *in vivo*, where the rich dermal vasculature is intact. By way of example, attention may be drawn to the fact that the GlucoWatch Biographer®, after its initial warm-up period, provides a measure of blood glucose every 10 minutes (23).

Internal standard calibration

As discussed before, the use of iontophoresis in glucose monitoring requires an initial calibration to be performed via a conventional ("finger-stick") blood measurement. Clearly, in situations for which less frequent monitoring is necessary, an alternative, noninvasive calibration procedure is essential. We have, therefore, considered the use of an internal standard calibration (Equation 2) (10). Endogenous electrolytes, such as sodium and potassium, seemed, *a priori,* good candidates as internal standards for lithium reverse iontophoresis. First, these are cations, extracted at the cathode and, like lithium, principally by electromigration. Second, their physiological concentrations are normally quite constant, ranging from 135 to 143 mM for sodium and between 3.3 and 4.6 mM for potassium (28).

To evaluate the idea, potassium and sodium were also quantified in all the reverse iontophoretic samples obtained in the fixed concentration and stepwise concentration change experiments discussed above and their extraction fluxes were determined. The essential requirement for the internal standard is that its extraction flux is independent of the target analyte concentration; in other words, that the transport number of the internal standard is constant and is not affected by variations in the analyte's subdermal level. This requirement was fulfilled by both sodium and potassium. Figures 2 (c) and (d) show the transport numbers of the three cations (Li^+, Na^+ and K^+) calculated using the J_{Li} data in Figure 2 (a) and 2 (b) and the corresponding measured values of J_{Na} and J_K. It is apparent that the sodium and potassium transport numbers were indeed constant within each experiment, while that of lithium varied as anticipated in proportion to the drug's subdermal concentration.

This finding is completely logical for sodium, which is present subdermally at a much higher concentration than lithium. When lithium transports a slightly greater or smaller amount of charge, due to its subdermal level changing, therefore, sodium (as the major current carrier) is able to "take up the slack" without a significant impact on its transport number (18). On the other hand, for potassium, whose physiological concentration is much closer to lithium's therapeutic range, one might have expected some compensation between the transport numbers of the two cations. That this is not the case may be explained by the fact that potassium is, in some respects, a more "efficient" charge carrier than sodium: that is, while sodium is 30 times more concentrated than potassium in the subdermal solution, the ratio of their extraction fluxes was only ~20, meaning that potassium has the significantly higher mobility of the two ions. It is not simply concentration, therefore, which determines the absolute value of a transport number. Unravelling the relationship between concentration, mobility, and other factors, which impact upon the manner in which the current is distributed among the available

charge-carriers, is an important future objective of iontophoretic research.

The next step was to verify the validity of Equation 2. The regressions shown in Figure 2 (e) and (f) demonstrate the correlations between the ratio of lithium to sodium extraction fluxes (R_{Na}) and either the lithium subdermal concentration, or the ratio of the subdermal concentrations of the two ions. When the corresponding regressions were performed for R_K, the correlations were satisfactory, though smaller ($r^2 = 0.89$ and 0.83, respectively), presumably because of the slightly higher variability observed in potassium fluxes as compared to those of sodium. Nevertheless, overall, this component of the study demonstrated unequivocally that the internal standard concept works *in vitro* and may constitute a viable approach to normalize lithium iontophoretic extraction for drug monitoring purposes.

C. Concentration-profile kinetic experiments

Further experiments examined whether transdermal iontophoresis could be used to determine pharmacokinetic parameters non-invasively. This was evaluated by simulating two classic plasma profiles in the subdermal compartment of the iontophoretic cells. It should be noted that the goal of these studies was to illustrate the versatility of reverse iontophoresis for non-invasive monitoring; there was no intention here to simulate "real" lithium pharmacokinetics, which can be complex (4, 29).

(a) IV bolus

The first kinetic profile considered was an IV bolus. Mono-compartmental kinetics were assumed. The model was characterized by an average volume of distribution of 4.54 ml (the mean subdermal compartment volume of diffusion cells) and a clearance of 1 ml/h (the syringe pump rate of perfusion). The dose "injected" was 4.96 µmoles. The subdermal concentration profile should therefore follow an exponential decay post-injection :

$$C_t = C_0.e^{-K_e.t} \qquad \text{Equation 4}$$

where C_t and C_0 are the lithium subdermal concentration at a given time t and at t=0, respectively, and K_e is the elimination rate constant. Upon combination of (i) Equations 3 and 4, and (ii) Equations 2 and 4, two testable hypotheses were defined for this experiment, namely:

$$J_{Li} = \gamma.C_0.e^{-K_e.t} \qquad \text{Equation 5}$$

$$R_{I.S.} = \gamma^{\#}.C_0.e^{-K_e.t} \qquad \text{Equation 6}$$

where the significance of J_{Li}, $R_{I.S.}$, γ and $\gamma^{\#}$ have been previously defined.

Throughout these experiments, sodium and potassium extraction fluxes were constant, averaging 9.4 µmol/h (± 4%) and 0.55 µmol/h (± 11%), respectively. On the other hand, the lithium fluxes, in general, tracked the changes in its subdermal concentrations. Figure 3 shows the lithium subdermal concentration, the lithium extraction flux and the ratios of extraction fluxes (J_{Li}/J_{Na} and J_{Li}/J_K) as a function of time post-injection. In general, good agreement with the model is observed. The subdermal concentration data allowed the reference values for the parameters characterizing the model to be determined (Table II). Clearance values estimated with both model-dependent and model-independent methods were very similar. These experimental parameters agreed well with the theoretical values cited above.

Figure 3 reveals that the values of J_{Li}, R_{Na} and R_K do not conform to the expected profiles (Equations 5 and 6) during the first hour of extraction. In fact, the profiles are reminiscent of an oral administration, for example, with a very fast absorption phase. After one hour, however, the three profiles decreased in parallel with the subdermal Li concentration. It

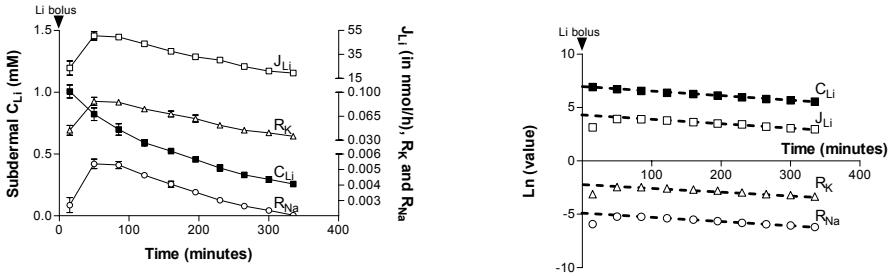

Figure 3: Simulated lithium bolus administration (left panel) and a semi-logarithmic transformation of the data (right panel). The solid squares are the subdermal lithium concentrations; the open squares are the iontophoretic extraction fluxes of the drug (J_{Li}). The open circles and open triangles are, respectively, the extraction flux ratios, R_{Na} and R_K. Each data point represents the mean ± standard deviation (n=6). Solid lines of simple interpolation through the data are shown in the left panel; the dashed lines in the right panel, on the other hand, are linear regressions.

appears therefore, that during the first hour, J_{Li}, R_{Na} and R_K reflect two concurrent processes: (i) the subdermal lithium kinetics, and (ii) the establishment of steady iontophoresis extraction fluxes across the skin. That is, until γ and $\gamma^{\#}$ become constant, the J_{Li}, R_{Na} and R_K profiles report on both processes. Once this is achieved, the extraction flux becomes directly and proportionally dependent on the subdermal concentration, and the slopes of the lines in Figure 3 are parallel.

Figure 4 presents the values of γ and $\gamma^{\#}$ as a function of time. A repeated measures ANOVA demonstrates that γ and $\gamma^{\#}$ become constant from the third sampling period (after 85 minutes of iontophoresis). Mean values for γ (68.8 ± 1.7 µl/h); $\gamma_{Na}^{\#}$ (7.3 ± 0.2 M^{-1}) and $\gamma_K^{\#}$ (114.1 ± 4.2 M^{-1}) were determined by linear regression through the data obtained post-85 minutes of iontophoresis.

Linear regression of the semi-logarithmic data (after the third sampling period) in Figure 3 permitted the elimination rate constant (K_e) to be determined from the iontophoretic extraction data and compared to the reference value (Table II). In general, K_e was slightly under-estimated relative to that calculated from the decay of the subdermal

Table II: Pharmacokinetic parameters determined in the bolus experiment (Mean ± SD, n=6).

	r^2 ≥	Ke ($.10^{-3}min^{-1}$)	$T_{1/2}$ (min)	C_0 [b,c] (mM)	Vd [b,c] (ml)	Cl [b,c] (ml/h)	Cl [b,d] (ml/h)
$C_{Li\ Subdermal}$	0.99	4.2 ± 0.3	168 ± 14	1.01 ± 0.06	4.9 ± 0.3	1.22 ± 0.05	1.21 ± 0.04
J_{Li}	0.99	3.9 ± 0.2	180 ± 10	0.99 ± 0.06	5.0 ± 0.3	1.17 ± 0.02	1.21 ± 0.03
R_K	0.96	3.6 ± 0.3[a]	193 ± 15[a]	0.97 ± 0.06	5.1 ± 0.3	1.10 ± 0.07[a]	1.21 ± 0.09
R_{Na}	1.00	3.8 ± 0.2	181 ± 9	0.98 ± 0.06	5.1 ± 0.3	1.17 ± 0.04	1.22 ± 0.06

[a] Value significantly different from reference value obtained from the direct measurement of C_{Li} ($P<0.05$) (Kruskal-Wallis ANOVA on ranks)

[b] Values determined using γ, $\gamma_K^{\#}$ or $\gamma_{Na}^{\#}$, respectively

[c] Calculated assuming a one-compartment model: $V_d=Dose/C_0$, $Cl=K_e.V_d$

[d] Model independent calculation using the corresponding area-under-the-curve (AUC)

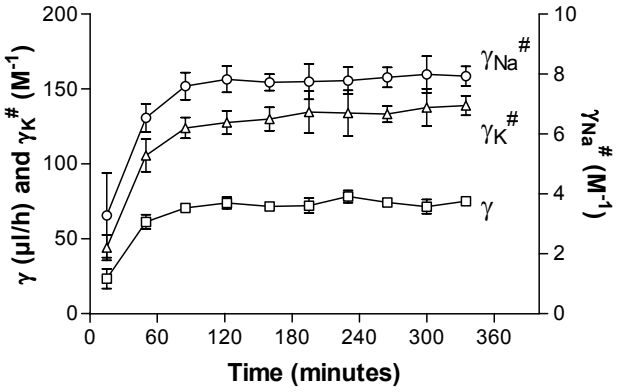

Figure 4: Proportionality constants γ and $\gamma^{\#}$ (defined in Equations 3 and 2, respectively, and determined from the bolus experiment) versus time. Each data point represents the mean ± standard deviation (n=6).

concentrations, resulting in a predicted half-life that was 13-25 minutes longer. Nevertheless, overall, the agreement was really quite good. One possible source of the differences observed is the time required for the iontophoretic sampling. The data point at 85 minutes, for example, reflects a moving average, so to speak, of the instantaneous values during the sampling period between 70 and 100 minutes. On the other hand, the subdermal C_{Li} was measured in a sample taken at exactly 85 minutes (i.e., the mid-point of the iontophoretic extraction period). An analogy may be drawn between measurements of urinary excretion rates and plasma concentrations. Clearly, when comparing kinetic parameters from the two types of measurement, the degree of error (difference) becomes greater as the sampling period increases. This fact has been recognized in the Glucowatch® with which glycemia is now assessed every 10 minutes such that very close tracking of glucose levels is possible. In the end, analytical sensitivity is the determining factor – for lithium, with the assay sensitivity and precision presently possible, 5-10 minute sampling intervals are feasible.

The iontophoretic extraction data were next used to estimate additional pharmacokinetic parameters (including the clearance (Cl) and volume of distribution (V_d)). Equations 5 and 6 indicate that C_0 can be found provided that γ and $\gamma^{\#}$, respectively, are known. Knowing C_0, it is then straightforward to assess V_d (=Dose/C_0) and Cl (=K_e.V_d). The first step, therefore, was to identify the values of γ and $\gamma^{\#}$ to be used. Figure 4 shows that these coefficients of proportionality become reasonably constant after about 1.5 hours of iontophoresis, and the mean values from this point on are the logical choices for the determination of the pharmacokinetic parameters of interest (Table II). The V_d and Cl determined in this way are quite close to the reference values. While this type of calculation based on *in vitro* data is usefully illustrative, it remains to be seen *in vivo* the extent to which γ and/or $\gamma^{\#}$ vary within and between subjects; that is, will it be necessary to determine γ and/or $\gamma^{\#}$ for every patient, or will a population average be sufficiently precise for all subjects? In the former ease, of course, careful calibration with blood sampling would be

necessary for each person in order to define the value of the constant(s) to be used.

Self-evidently, the internal standard calibration approach leading to the deduction of $\gamma^{\#}$ is envisaged as a means to completely avoid calibration with blood sample. The constancy of Na^+ extraction in this work speaks to its considerable potential in this regard; on the other hand, potassium, which was also evaluated, yielded more variable results. Certainly with Na^+, then, the approach could be useful for a therapeutic monitoring application during lithium therapy. Care is necessary with respect to the kinetics, however, as a finite time is required before γ and $\gamma^{\#}$ reach stable values (Figure 4). Whether this delay is partly an artefact, caused by the experimental design in which Na^+ and K^+ extraction fluxes were stabilized before Li was "injected" into the subdermal solution, remains to be seen. In a recent study examining the idea of valproate monitoring by reverse iontophoresis, and using glutamate as an internal standard, the value of $\gamma^{\#}$ was constant from the very first sampling period, even though the extraction fluxes had not stabilized by this time. It is important to note, in this case, that the two ions were introduced simultaneously into the subdermal compartment. It follows that, for a patient receiving chronic lithium therapy, it is reasonable to expect $\gamma^{\#}$ to become constant more rapidly than that observed in the *in vitro* work presented here.

(b) Constant rate infusion

The second situation considered was a constant infusion (5.6 μmoles/hour) of lithium chloride into the subdermal compartment having V_d ~ 4.54 ml. The clearance (syringe pump rate of perfusion) was 4 ml/h. The drug concentration profile, in this case, is described by the Equation 7 (27, 30):

$$C_t = C_{ss}(1 - e^{-Ke\,t}) = \frac{K_0}{Ke\,V_d}(1 - e^{-Ke\,t}) \qquad \text{Equation 7}$$

which predicts that C_{Li} will increase exponentially to a steady-state plateau, C_{ss}. Figure 5 shows that this plateau level (~1.3 mM) was attained after 3-3.5 hour of perfusion. When the subdermal concentration

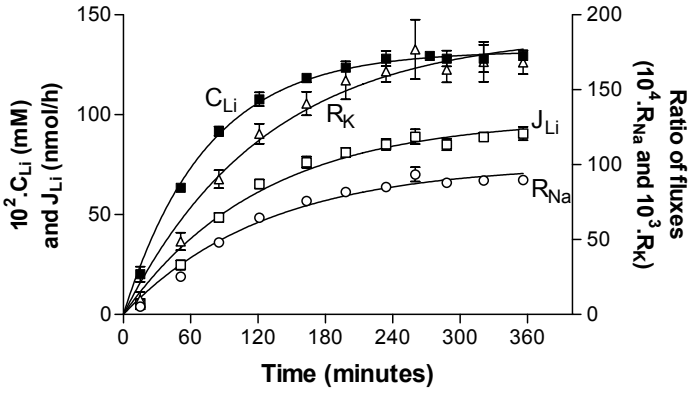

Figure 5: Iontophoretic monitoring of lithium during a simulated constant infusion to steady-state. The subdermal lithium concentration profile is represented by the solid squares and the line through the data is a fitted curve according to Equation 7; the open squares are the iontophoretic extraction fluxes of the drug (J_{Li}) (data fitted to Equation 8). The open circles and open triangles are, respectively, the extraction flux ratios, R_{Na} and R_K (results fitted using Equation 9). Each data point represents the mean ± standard deviation (n=6).

profile was fitted to equation 7 ($r^2 \geq 0.94$, non-linear regression), the following reference values were obtained; K_e = 0.014 ± 0.001 min^{-1}; $T_{1/2}$ = 50 ± 4 min; C_{ss} = 1.32 ± 0.03 mM; V_d = 5.1 ± 0.3 ml; and Cl = 4.2 ± 0.1ml/h.

Figure 5 also demonstrates that the iontophoretic extraction of Li flux and the extraction flux ratios (R_{Na} and R_K) also increased exponentially towards steady-state values, conforming to the following equations which result from the substitution of Equations 3 and 2, respectively, into Equation 7:

$$J_{Li} = J_{ss}.(1-e^{-K_e.t}) = \gamma.C_{ss}.(1-e^{-K_e.t}) \qquad \text{Equation 8}$$

$$R_{I.S.} = R_{ss}.(1-e^{-K_e.t}) = \gamma^{\#}.C_{ss}.(1-e^{-K_e.t}) \qquad \text{Equation 9}$$

The significance of $J_{Li,}$ $R_{I.S.}$, γ and $\gamma^{\#}$ are as before; J_{SS} and R_{SS} represent the steady-state values of J_{Li} and $R_{I.S.}$, respectively.

Interpretation and analysis of the iontophoretic extraction flux data were more complicated than the IV bolus case. In the latter situation, the only "pharmacokinetic" process taking place in the subdermal compartment is drug elimination. In contrast, in the infusion scenario, there is both "input" and elimination; it should be recalled, furthermore, that the time to steady-state under these circumstances is on the order of four elimination half-lives. This relatively slow approach to steady-state means that the biphasic behaviour of J_{Li} and $R_{I.S.}$ seen in the I.V. bolus situation is "lost" when the drug is perfused into the subdermal compartment. It is not that the biphasic phenomenon is no longer occurring, it is simply that the data are not sufficiently precise, nor were they acquired at a high enough frequency, to allow this behaviour to be observed. This conclusion is confirmed somewhat by the fact that fitting Equations 8 and 9 to the data in Figure 5 is much better when the two first samples are omitted; after about an hour, however, the model fits the results very well.

The evolution of γ and $\gamma^{\#}$ with time is shown in Figure 6. It is first noted that the absolute values of these parameters are very similar to those observed in the I.V. bolus experiments (Figure 4), supporting the contention that these proportionality constants may show very small inter-individual differences. Also clear when comparing Figures 4 and 6, however, is that it takes longer for the values of γ and $\gamma^{\#}$ to stabilize in the

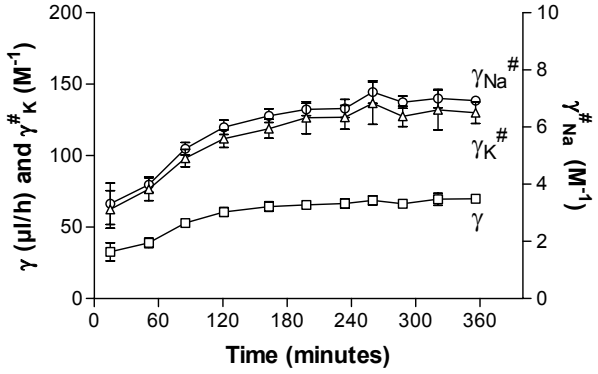

Figure 6: Proportionality constants γ and $\gamma^{\#}$ (defined in Equations 3 and 2, respectively, and determined from the constant infusion experiment) versus time. Each data point represents the mean ± standard deviation (n=6).

infusion situation; a repeated measures ANOVA indicates that 2.5-3 hours are necessary, in fact. At this point, it was deduced that: γ=65 ± 14 µl/h, $\gamma_K^{\#}$ =135 ± 43 M^{-1}, and $\gamma_{Na}^{\#}$ =7.6 ± 1.4 M^{-1}. It is logical to suppose that the increased time for stabilization is due to the same phenomenon implicated in the I.V. bolus case, superimposed upon the fact that steady-state kinetics are approached only quite slowly when the drug is infused into a compartment from which clearance is not rapid.

The iontophoretic extraction fluxes become progressively more reflective of the subdermal kinetics with increasing time, therefore. When the iontophoretic data in Figure 5 are fitted to Equations 8 and 9, the values of $J_{S.S.}$, $R_{S.S.}$ and K_e obtained are very close either to the experimental results or to the theoretical value. Similarly, the derived values for clearance (Cl) are in excellent agreement with the reference value of 4.2 ± 0.1 mL/h. From the stable values of γ and $\gamma^{\#}$, the calculated clearances were 4.1 ± 0.1 (from J_{Li}), 4.1 ± 0.2 (from R_K) and 4.1 ± 0.1 ml/hour (from R_{Na}).

Estimation of K_e from the iontophoretic extraction data was more challenging. This parameter was obviously underestimated when data from the earliest sampling times were included (Figure 7). When these initial results were omitted, the fitting procedure improved ($r^2 \geq 0.8$); for example, analysis of data from 85 minutes onward resulted in values of K_e (in min^{-1}) of 0.0095 ± 0.0003 (from J_{Li}), 0.0091 ± 0.0009 (from R_K) and 0.0091 ± 0.0005 (from R_{Na}). The "reference" value, it is recalled, was 0.014 min^{-1}. Thereafter, if results only from the latter half of the experiment are fitted (Figure 7), the resulting K_e increased somewhat but not significantly, and the goodness-of-fit decreased. It is not clear whether, in this experiment, the iontophoretic fluxes could ever provide a better estimation of K_e. Once steady-state is achieved, of course, the sensitivity of the model to determine K_e is lost. It follows that there is a temporal "window of opportunity" for the estimation of K_e; long enough has to have elapsed so that the extraction fluxes have caught up with the

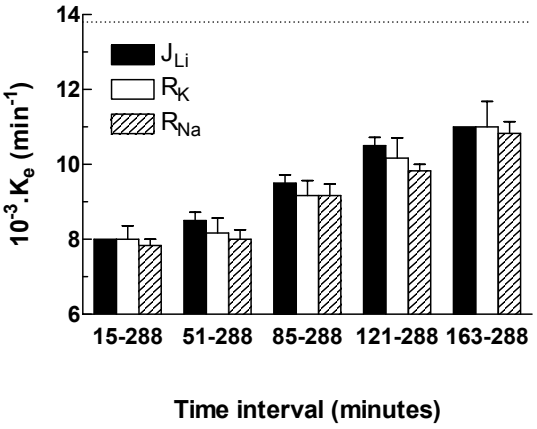

Figure 7: Values of K_e (mean ± SD; n=6) determined from the constant infusion experiment. Data for J_{Li}, R_{Na} and R_K (filled, open, and hatched bars, respectively) were fitted to Equations 8 and 9. The results were calculated from the individual data obtained during the different time intervals indicated on the abscissa. The dashed line at K_e = 0.014 min^{-1} indicates the "reference" value of this parameter.

subdermal kinetics, but not so long that steady-state is close to having been attained. Clearly, in this situation, the practical application to pharmacokinetic profiling will be limited. On the hand, the results demonstrate an effective means with which to determine noninvasively, and quite accurately, a drug's clearance.

In conclusion, the results presented here demonstrate that reverse iontophoretic monitoring of lithium is concentration-dependent, and that quantitative information about the drug's subdermal level can be obtained without a "blood" measurement via the use of Na^+ and/or K^+ as an "internal standard". The iontophoretic extraction flux of Li, and the ratio of this flux to that of either Na^+ or K^+, tracks pharmacokinetic changes in the drug's subdermal concentration rapidly and faithfully, allowing the remarkably noninvasive determination of certain pharmacokinetic parameters that are presently available only via plasma or whole blood measurements.

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Notations

J	Extraction flux (units: nmol/h)
$R_{I.S.}$	Ratio between lithium (Li) and internal standard (I.S.) extraction fluxes ($J_{Li}/J_{I.S.}$)
C_{Li}	Lithium subdermal concentration (units: mM)
γ	Proportionality constant (units: µl/h) between J_{Li} and C_{Li}
$\gamma^{\#}$	Proportionality constant (units: M^{-1}) between $R_{I.S.}$ and C_{Li}
t_a	Transport number of ion a
K_e	Elimination rate constant (units : min^{-1})
$T_{1/2}$	Half-life (min)
C_t	Concentration at a given time t (mM)
V_d	Volume of distribution (ml)
Cl	Clearance (ml/h)

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Chapitre 4

Le monitoring non-invasif du lithium par ionophorèse inversée Une étude in vivo

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Résumé

Buts : Cette étude évalue *in vivo* les capacités de l'ionophorèse inversée à devenir une méthode non-invasive de monitoring de la lithiémie et une alternative aux prélèvements sanguins. 23 patients souffrant de troubles bipolaires ou schizo-affectifs et sous lithiothérapie ont participé à cette étude.
Méthodes : L'ionophorèse, réalisée sur l'avant-bras des volontaires, fut bien acceptée. Les échantillons ionophorètiques ont été analysés pour quantifier les ions lithium, sodium, potassium et calcium extraits à la cathode par électromigration. Un prélèvement sanguin classique a été prélevé afin de fournir les valeurs de référence du lithium et des autres électrolytes.
Résultats : Les flux d'extraction du lithium varient proportionnellement avec les lithiémies sériques. En revanche, ceux du sodium, potassium et calcium étaient relativement constants. Aussi, ces électrolytes furent considérés comme de possibles standards internes pour la calibration de la méthode de monitoring du lithium. Il a été observé que le rapport des flux d'extraction était proportionnel à la lithiémie sérique et que seul le sodium, se comportait comme un standard interne acceptable. La population a ensuite été partagée aléatoirement en deux groupes distincts. Les constantes d'extraction ionophorétiques ont été déterminées d'après le premier groupe et utilisées pour prédire les lithiémies sériques du second groupe. La comparaison des valeurs prédites et des valeurs de références a démontré une excellente capacité prédictive.
Conclusions : Cette étude prouve que l'ionophorèse inversée offre une méthode alternative et non-invasive pour le monitoring de la lithiémie.

Mots clés : Ionophorèse, Monitoring thérapeutique, Lithium, Extraction transdermique, Non-invasive

Lithium monitoring by reverse iontophoresis in vivo

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Abstract

Reverse transdermal iontophoresis was investigated as an alternative and non-invasive method for lithium monitoring *in vivo*. Experiments were performed in 23 bipolar or schizo-affective patients. Lithium, sodium, potassium and calcium were efficiently extracted by iontophoresis. A conventional blood sample provided comparative reference values for the drug and other electrolytes. Lithium extraction fluxes were proportional to the corresponding serum concentrations. In contrast, sodium, potassium and calcium extraction fluxes were relatively constant, consistent with their stable blood levels. Normalization of the lithium extraction flux with that of sodium, which acted as an "internal standard", permitted calibration of the monitoring procedure without the need for a blood measurement. This conclusion was then tested retrospectively by dividing the patient population into two groups of equal size. The reverse iontophoretic extraction data from the first subset were used first to establish the proportionality between lithium iontophoresis (or the relative electrotransport of Li and Na) and the lithium blood level (or the ratio of Li to Na systemic concentrations), and then second to predict lithium blood levels in the second subset of patients. An excellent predictive ability was achieved, with the use of the internal standard significantly reducing the confidence interval associated with the predicted value. In conclusion, reverse iontophoresis appears to offer a novel and accurate method for lithium monitoring.

Keywords: Iontophoresis, Therapeutic drug monitoring, Lithium, Transdermal extraction, Non-invasive

I. Introduction

Fifty years after the first report of its anti-manic effects (1), lithium remains a first choice mood stabilizer for preventing relapses in bipolar disorders (1-3). It is also the only mood stabilizer for which a preventive effect on suicidal risk has been clearly shown (3,4). Regular lithium monitoring is essential to ensure efficacy and to prevent adverse effects (5). While steady-state plasma levels of the drug are achieved in a few days, a therapeutic response, in the treatment of manic episodes for example, is not observed until the two to three weeks post-initiation of treatment (6). Serum levels are monitored, therefore, on a weekly basis at the beginning of the treatment, then monthly or even longer intervals (7). More frequent supervision is required (i) in the absence of a satisfactory therapeutic response, (ii) in the presence of adverse effects or disease that affect drug disposition, (iii) to verify compliance (~40% poor compliance has been reported for bipolar patients (8)), or (iv) when using poly-pharmacotherapy involving potentially interacting drugs (9). Lithium monitoring involves a blood test which provides the so-called "Standardized 12 hour Li^+ Serum Concentration". That is, patients are subjected to a blood draw in the early morning, approximately 12 hours after the last dose of the day before, and prior to the first administration on the day of monitoring.

It can be argued, therefore, that a completely non-invasive technique for lithium monitoring would facilitate compliance with treatment and improve the quality of life for bipolar patients. Saliva has been considered as an alternative matrix for lithium monitoring, but the broad inter and intra-individual variability of the saliva/plasma concentration ratio severely limits the usefulness of this approach (10,11). The skin offers, in principle, an extensive and accessible surface for drug sampling, and early research demonstrated the transdermal efflux of substances into collection patches by passive diffusion and secretion in the sweat (12-14). However, because these mechanisms are slow and inefficient, the long accumulation time required before the drug can be detected in the patch renders the method impractical for pharmacokinetic tracking (15).

Transdermal flux can be significantly increased, however, by iontophoresis in which a low electrical current (<0.5 mA/cm^2) is applied to the skin to facilitate polar and charged molecular transport through the barrier (16). Initially developed for transdermal drug delivery, iontophoresis has also been investigated as an alternative, non-invasive sampling technique. Indeed, The U.S. Food and Drug Administration recently approved a "reverse" iontophoretic device (GlucoWatch Biographer®, Cygnus Inc., CA, USA) that monitors glycaemia for 12 hours to help in the management of diabetes (17). The potential application of this technique for the monitoring of drugs and clinical markers has been recently reviewed (18). Two mechanisms of transport are involved in iontophoresis. Electromigration concerns the movement of ions, which carry charge across the skin and are driven (attracted) specifically towards the electrode of opposite polarity. Electroosmosis is a net solvent flow in the anode-to-cathode direction, which enables much improved permeation of neutral species (e.g., glucose), and further enhances cationic transport (19).

Lithium monitoring by reverse iontophoresis has been investigated *in vitro* (20). This small, mobile and cationic drug is an excellent candidate for iontophoretic extraction. Lithium is transported by electromigration to the cathode (negative electrode), at a flux (J_{Li}, nmol/h) described by equation 1:

$$J_{Li} = \frac{I.t_{Li}}{F.Z_{Li}} \qquad \text{Equation 1}$$

t_{Li} and z_{Li} are, respectively, the drug's transport number and valence (+1), F is Faraday's constant, and I is the current applied. The

transport number of a given ion (i.e. the fraction of charge transported through the skin by that ion) is proportional to its concentration when competing co-ions are present (21). This is obviously the case in reverse iontophoresis where the physiological milieu provides a panoply of competing co-ions. Under these circumstances, Equation 1 can be alternatively expressed as:

$$J_{Li} = \gamma . C_{Li} \qquad \text{Equation 2}$$

where C_{Li} is the subdermal concentration of lithium and γ is a constant. The *in vitro* investigation demonstrated a linear relationship between J_{Li} and C_{Li} (20). *In vivo*, on the other hand, it is important to correlate J_{Li} with the serum concentration of the drug. While it is perhaps reasonable to suppose that the subdermal compartment is in rapid (or even instantaneous) equilibrium with the blood for a hydrophilic compound, such as lithium, it was nevertheless an important initial objective of this work to verify this hypothesis. To this end, only subjects undergoing chronic lithium therapy were admitted into the study, ensuring that any impact of slow drug distribution kinetics would be minimized.

Iontophoresis is not a selective extraction process; in fact, all the ions present in the system compete for transporting the charge (22). Only those ions with a sufficiently high transport number will be efficiently extracted. The competitive nature of the extraction process and the complex composition of the "*in vivo*" milieu mean that transport numbers cannot be predicted *a priori*. If the efficiency of lithium extraction (characterized by the constant γ in Equation 2) shows wide inter- and intra-subject variability, a blood level calibration is obligatory. Such is presently the case for Glucowatch Biographer®, for example. In the case of therapeutic lithium monitoring, of course, reverse iontophoresis offers no advantage if calibration in this way is necessary. Additional objectives of the present study, therefore, were, on the one hand, to examine the degree to which γ varies between subjects and, on the other, to explore an "internal standard" concept (20,23,24) as a non-invasive means with which to standardize the reverse iontophoresis of lithium.

An internal standard (I.S.) is defined as an endogenous compound which is present at relatively constant systemic concentration. It follows that the ratio of extraction fluxes $J_{Li}/J_{I.S.}$ should be directly proportional to the ratio of subdermal concentrations, $C_{Li}/C_{I.S.}$ In other words, and given that $C_{I.S.}$ is constant:

$$J_{Li}/ J_{I.S.} = \gamma^{\#} . C_{Li} \qquad \text{Equation 3}$$

Thus, the simultaneous, reverse iontophoretic extraction of Li and I.S. enables the concentration of the analyte of interest to be directly determined once $\gamma^{\#}$ is known. The validity of this hypothesis has been demonstrated *in vitro* for lithium, using sodium and potassium as internal standards (20). Here, the internal standard calibration has been tested *in vivo*, and the suitability of the major cationic electrolytes: sodium, potassium, calcium, magnesium and ammonium, has been evaluated.

II. Methods

A. Materials

L-histidine (USP, Eur.Ph.), sodium chloride (Eur.Ph.), silver wire 99.9%, silver chloride 99%, and platinum 99.9% were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Deionized water (resistivity $\geq$ 18.2 MΩ.cm) was used to prepare all solutions.

B. Subjects

Thirty ambulatory patients, diagnosed with bipolar or schizo-affective disorder, entered the study. The subjects were receiving chronic lithium therapy, at doses between 12 to 36 mmol of Li per day (sulfate or carbonate

salt), for no less than 3 weeks. Of the original subjects enrolled, data from 6 patients were unusable (due to current interruption during iontophoresis, or to a leak from the collection chamber, or because of a missing blood sample). The results presented below, therefore, correspond to 24 patients (11 women, 13 men), aged 20 to 59 years.

The clinical protocol was accepted by the internal review board of the Geneva University Cantonal Hospital. Informed consent was obtained from all participants. Patients arrived at 08.00 hr for their regular Li monitoring and were maintained in supine position throughout the study. The arm presenting the better venous accessibility was reserved for the standard monitoring procedure. The other forearm was used for reverse iontophoresis. A glass cylinder, which served as the cathodal compartment, was fixed to the forearm (at a site which had been cleaned with an alcohol swab) via a Teflon ring. Silicone grease was applied to avoid leaks. The ensemble was held firmly in place with medical tape (3M 9772L Foam Tape, 3M Health care, St Paul, MN, USA). The area of transport through the skin was 3.2 ± 0.4 cm^2. The glass cell was filled with 1.2 mL of a 10 mM histidine aqueous solution (pH = 7.47) for 1 minute ; this liquid was then removed and refreshed and a Ag/AgCl electrode was then inserted into the solution and maintained at least 5 mm from the skin surface via a plastic top that covered the cell.

The anodal chamber was either a similar glass cell, again equipped with a Ag/AgCl electrode, and filled with 62 mM NaCl solution, or a 32 cm^2 commercially-available adhesive iontophoretic patch (Dispersive Pad, Iogel Medium, Iomed, Salt Lake City, Utah, USA). The anodal electrode was applied on the same forearm at approximately 10 cm from the glass cell holding the cathode.

A Phoresor II Auto (PM850, Iomed) was used to deliver a constant direct current of 0.8 mA. Four iontophoretic intervals of 30 minutes were performed. At the end of each interval, the current was stopped and the electrode solutions were entirely removed and refreshed. The electrode solutions were analyzed for lithium and other cations as described below.

A blood sample was taken between 90 to 100 minutes post-initiation of iontophoresis and was reserved for subsequent analysis (see below).

C. Analytical methods

Ionic chromatography was used to quantify lithium, sodium, potassium, calcium and magnesium in the electrode chamber solutions. A Dionex DX-600 (Dionex, Voisins le Bretonneux, France) equipped with a GP-50 pump, a AS-50 thermostated compartment (40°C), an ED-50 detector, a CS-16 cationic column and an Atlas suppressor was used. Data analysis was performed with a Chromeleon V.6.4 software (Dionex). Lithium, sodium and potassium were separated with a 6.25 mM HMSA mobile phase flowing at 2 ml/min and a suppressor current of 42 mA). Calcium and magnesium were separated with a 25 mM HMSA mobile phase, a 1mL/min flow rate and a suppressor current of 83 mA).

Lithium was quantified in the blood samples with an ion selective electrode (ISE) (Cobas Integra Model 700, Roche Diagnostics, Basel, Switzerland). Sodium, potassium and calcium were also quantified via ISEs (Synchron LX20 Beckman Coulter Inc, Fullerton, CA, USA). Magnesium and ammonium were assayed (Synchron LX20) with colorimetric and enzymatic tests, respectively.

D. Statistics

All data are shown as the mean ± standard deviation unless otherwise indicated. Data analysis and linear regression were processed with Graph Pad Prism V.3.02 (GraphPad

Software, Inc., San Diego, California, USA). Repeated measures ANOVAs were use to compare ion fluxes at different times. The level for statistical significance was set at $P<0.05$. All the regressions presented in this work were significant ($P<0.0001$) unless otherwise stated. Confidence intervals were calculated as described elsewhere (25).

III. Results & Discussion

The serum concentrations of lithium, sodium, potassium, magnesium, calcium and ammonium are presented in Figure 1. Lithium levels fell in the range typical of patients being treated at the Geneva University hospital, with about 75% having steady-state concentrations between 0.5 and 0.9 mM. The rest had lower levels, considered to be out of the therapeutic range, presumably due to poor compliance. Almost all serum concentrations of the other cations fell inside the normal physiological range. The coefficients of variation were 1.7% for sodium, 6.1% for potassium, 6.2% for magnesium, and 3.6% for calcium. The corresponding figure for ammonium was over 65%; consequently, no further attention was focused on this species.

The extraction procedure began with a 1-minute "washing" period during which current was not applied. This "washing" step serves as an indicator of the existence (or not) of ion reservoirs in the more superficial layers of the skin (a point discussed further below). All "washing" samples contained detectable amounts of sodium and potassium, while lithium and calcium were detected in 22 and 16, respectively, of the 24 samples analyzed. Magnesium was rarely detected (only 2 samples). The amounts recovered (in nmol) were: lithium 14.4 ± 14.2, sodium 163 ± 212, potassium 106 ± 140, and calcium 10.5 ± 10.2.

Iontophoresis was well-tolerated by the subjects. Pricking sensations were frequently reported, being more noticeable at the anode at the start of each current interval. A mild, uniform and transient redness (typically resolved within a couple of hours) was observed at both electrode sites at the end of the protocol, similar to that which has been observed in previous work (26,27).

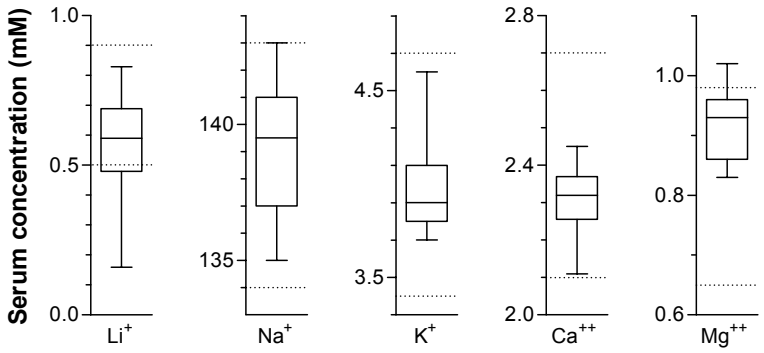

Figure 1: Box and whisker representation of the measured serum levels of lithium, sodium, potassium, calcium and magnesium for 24 subjects. Dotted lines indicate the "normal" range observed (28). Note that the range for lithium is that currently adopted by the Department of Psychiatry of the Geneva University Hospital.

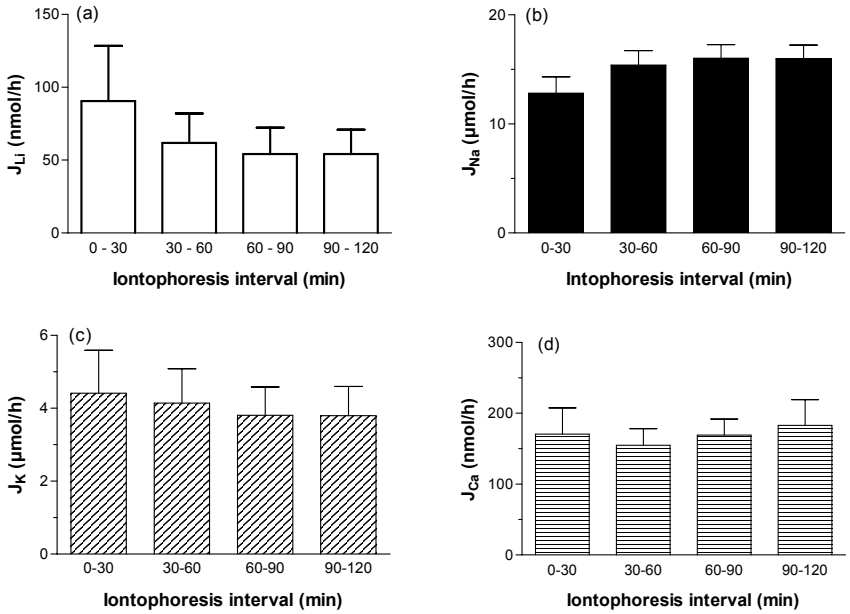

Figure 2: Iontophoretic extraction fluxes (mean±SD; n=22 or 23) of lithium (J_{Li}), sodium (J_{Na}), potassium (J_K) and calcium (J_{Ca}) at each iontophoretic sampling interval. For J_{Li} and J_{Na}, the 0-30 value is significantly different from each of the subsequent samples. For J_K, the 0-30 value differs significantly from the two last samples. For J_{Ca}, the 0-30 value differs significantly from 30-60 sample which, in turn, differs significantly from the last measurement.

A. Ionic fluxes and transport numbers

Lithium was efficiently recovered at the cathode for all patients. In the first six subjects, the anode chamber was also analyzed for lithium and, although a measurable amount was found, the calculated flux never exceeded 6% of that to the cathode. Subsequently, for practical reasons, disposable electrode patches were used for the anode in the remaining patients.

Figure 2 shows the reverse iontophoretic extraction fluxes measured for each cation in the four successive 30-minute periods of current passage. The average lithium flux (Figure 2(a)) was 90.5 ± 37.9 nmol/h for the first sampling interval, and was significantly higher ($P< 0.001$) than those measured subsequently (average = 56.7 ± 18.6 nmol/h). This higher initial extraction, plus the fact that lithium was present in the pre-treatment "washing" solution, suggests the existence of a lithium reservoir in the skin and/or skin appendages. Lithium has been found in cutaneous lesions (rashes and ulcers) and in biopsies obtained from the epidermis, dermis and subcutaneous adipose tissue of patients and guinea pigs chronically-treated with the drug (29,30). It has also been demonstrated that lithium is secreted in sweat (31,32); thus, lithium found in the "washing" samples could also originate from this source. This apparent accumulation of lithium into the skin may lead to the creation of a reservoir, the magnitude of which is unrelated to the drug's serum concentration. Depletion of this reservoir would explain the early higher fluxes, and the

fact that the extraction then stabilizes at a constant, lower level. *In vitro*, this phenomenon has not been observed (20); in that study, the skin used was obtained from pigs that had never been exposed to the drug. Under these circumstances, the extraction flux, not unexpectedly, increases towards its steady value. Parenthetically, it is worth noting that a skin reservoir has also been identified for glucose (in this case due, it is believed, to metabolic breakdown of skin lipids) and that it is therefore necessary to deplete this material before calibration and operation of the Glucowatch® (27).

Sodium extraction showed a different pattern (Figure 2(b)). The flux in the first interval (12.9 ± 1.6 µmol/h) was significantly less ($P < 0.001$) than the subsequently stable value achieved (15.8 ± 1.3 µmol/h). Potassium transport slightly but significantly ($P \leq 0.001$) decreased from 4.4 (±1.2) to 3.8 (±0.8) nmol/h; the flux of this cation was more variable than sodium: coefficients of variation were 21% and 8%, respectively, for the last three sampling periods. Calcium extraction did not evolve in any clear pattern, the flux decreasing from 172 (± 37) nmol/h at 30 minutes to 156 (± 24) at 60 minutes, and finally reaching 185 (± 37) nmol/h at 2 hours. The coefficient of variation associated with calcium transport was also high (18%). Finally, magnesium extraction fluxes, while detectable, were below the limit of quantification.

The *a priori* prediction of transport numbers remains challenging, particularly in reverse iontophoresis where the spectrum of potential charge carriers from within the body to the skin surface is very broad. In general terms, though, it is known that the transport number of a specific ion will depend on its concentration and mobility, relative to the concentrations and mobilities of the competing ions in the system (22). For this reason, of course, sodium is by far the principal charge carrier, being a mobile cation present at a much higher concentration than all others. Its transport number ($t_{Na} = 0.54 \pm 0.04$) and that of lithium ($t_{Li} = 0.0018 \pm 0.0006$) were quite consistent with the corresponding values determined *in vitro* (20), in an experiment designed to mimic, to some extent, the *in vivo* situation examined in this work. The transport number of potassium ($t_K = 0.13 \pm 0.03$) was higher than that measured *in vitro* (0.042 ± 0.006) (20), but was consistent with other values previously determined *in vivo* (33).

B. Relationship between lithium serum concentrations and iontophoretic extraction fluxes

A key practical objective of this study was to establish whether a clear relationship existed between lithium systemic levels and reverse iontophoretic extraction rates across the skin. Figure 3 illustrates how the serum lithium concentrations, measured approximately 90 minutes after the initiation of current passage, compared with the drug's iontophoretic fluxes for the four different, 30-minute periods of extraction. Linear regression of these data resulted in the correlations in Table I. While it is clear that there is a very poor relationship between the serum levels and the extraction data from the first sampling period, the correlations thereafter are excellent.

The initially poor correlation is probably due, in large part, to the skin reservoir of the drug discussed above, and the fact that the amount of drug "stored" in the skin is not related to the instantaneous serum level. It is necessary, therefore, for reverse iontophoresis to first "empty" this reservoir before it can reliably inform on the systemic concentration of the drug. While it is certainly logical to expect that the iontophoretic flux should correlate better at times closer to the blood sampling, the terminal half-life of lithium (14 hours or more (5)) is sufficiently long that the concentration 30 minutes post-initiation of

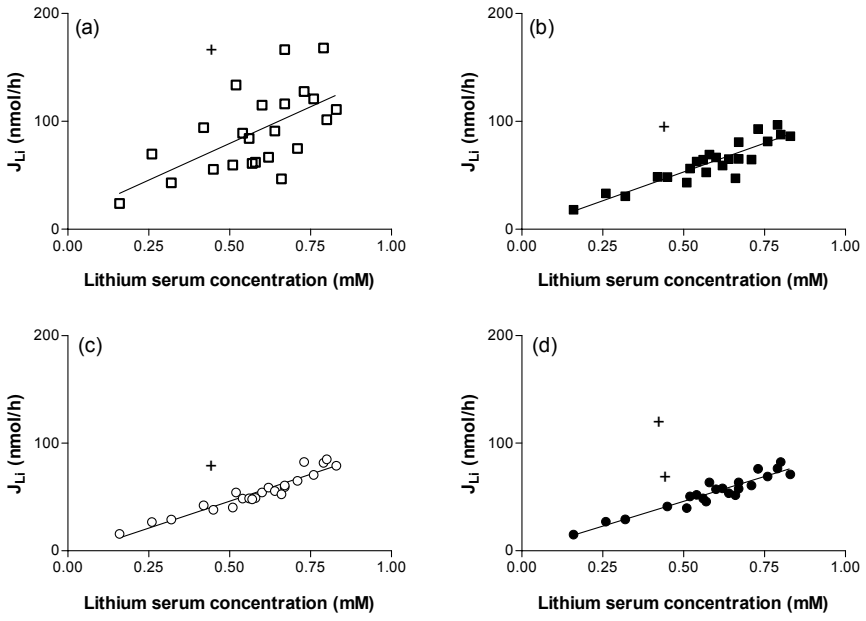

Figure 3: Reverse iontophoretic extraction fluxes of lithium at (a) 30 minutes, (b) 60 minutes, (c) 90 minutes, and (d) 120 minutes, plotted as a function of the corresponding serum concentration measured at 90-100 minutes post-initiation of current flow. Lines of linear regression are drawn through the data (Equation 2), and the results of this analysis are in Table I.

reverse iontophoresis would only be slightly different from that an hour later, and unquestionably not so divergent as to explain the poor correlation with the first period of iontophoresis relative to the later measurements.

Nevertheless, the results in Figure 3 and Table I are overwhelmingly positive in terms of establishing the *in vivo* relationship proposed between lithium levels and reverse iontophoretic extraction flux. After an hour of current passage, excellent correlations were

Table I: Linear regressions, at different times of iontophoresis, of (i) the extracted lithium flux (J_{Li}) (data in Figure 3), and (ii) the ratio of extracted lithium and sodium fluxes (J_{Li}/J_{Na}), as a function of the serum drug levels, according to Equations 2 and 3, respectively. Results are based on data from 23 patients.

	J_{Li}			J_{Li}/J_{Na}		
Time (min)	$\gamma \pm$ SE (µl/h)	Intercept (nmol/h)	r^2	$\gamma^{\#}_{Na} \pm$ SE (M^{-1})	Intercept	r^2
30	136 ± 38	11.5	0.38	11.7 ± 3.0	$4.\ 10^{-4}$	0.42
60	107 ± 11	-0.3	0.82	6.9 ± 0.4	$5.\ 10^{-5}$	0.93
90	100 ± 7	-4.0	0.91	6.1 ± 0.4	$-2.\ 10^{-4}$	0.92
120	92 ± 7	-0.2	0.90	5.6 ± 0.3	$7.\ 10^{-5}$	0.93

obtained, with data both from patients with "normal" drug concentrations, within the therapeutic window, and from those with low lithium levels. The challenge for a practical monitoring system will include, self-evidently, minimizing the time necessary to deplete the skin's reservoir of lithium, and shortening as far as possible the time of current passage necessary for the reliable and sensitive analysis of the drug in the collection device.

Figures 3(c) and 3(d) highlight statistically aberrant data (Grubbs's test, $P < 0.01$), which were not used in the overall statistical analyses presented here, from two patients (for this reason, the number of subjects indicated in the results is sometimes slightly different). The reverse iontophoretic extraction flux from one of these subjects was exceptionally high during the two later periods of iontophoresis; for the other volunteer, only the result in the final period was abnormally large. Precise explanations for these observations are not available. Perhaps, the former patient had an exceptionally large skin reservoir of the drug; possibly, the outlier for the second patient was the result of an accidental contamination. Clearly, additional studies, in much larger populations, will ultimately be necessary to provide information about intra- and inter-subject variability, and potential sources of systematic and opportunistic error.

The linear correlation between lithium extraction fluxes and the corresponding serum levels provides a quantitative measure of the proportionality constant (γ) linking the two variables (Equation 2). The most direct evidence of the potential of reverse iontophoresis for lithium monitoring would be to demonstrate that γ varies very little both between different individuals and within each person tested. That is, it would be possible then to derive a "global" value of γ that could be used in all patients, with high confidence, to convert their reverse iontophoretic extraction flux to a serum concentration. The correlation based on the first 30-minute period of extraction would obviously be unacceptable (see Figure 4 and Table I): γ has a 28% coefficient of variation (CV). However, for the next extraction period, the CV is down to 10% and, in the two final intervals, it falls further to around 7%. Compared to glucose extraction via the electroosmotic flow induced by iontophoresis, this level of variability in the proportionality constant is extremely low and reflects the relative stability of the transport numbers of the competing cations as described earlier. In other words, the fact that the

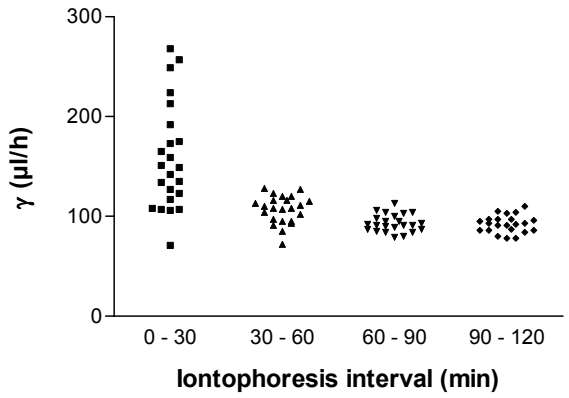

Figure 4: Experimentally determined values of γ, the proportionality constant between J_{Li} and C_{Li} (Equation 2) as a function of the duration of iontophoresis.

concentrations and mobilities of these ions are reasonably constant *in vivo* (or at least within sufficiently limited ranges such that their electrotransport kinetics are not too variable) means that the lithium extraction rates will respond to differences in the serum level in a rather consistent fashion from patient to patient. If confirmed in a much larger group of subjects, the variability in γ may, in theory, be small enough to provide a general "calibration factor" for the direct therapeutic monitoring of lithium by reverse iontophoresis.

C. Internal standard calibration

Recognizing, however, that the straightforward approach proposed above carries with it an obvious risk of data verification, the "internal standard" calibration idea was examined. The value of this strategy had been previously demonstrated *in vitro*, using as potential "internal standards", the sodium and potassium cations. Physicochemically much like lithium, these ions are small and mobile and are principally transported across the skin by electromigration.

A key criterion for any one of these cations to act as an "internal standard" for lithium is that their iontophoretic extraction fluxes be constant. For sodium, this was indeed the case: its transport number, as the principal charge carrier, was high (~0.54), with a CV of around 8%. In contrast, potassium and calcium revealed much more variable values for their transport numbers with CVs between 15 and 25%. Again, it is not entirely clear why such wide variability should have been seen. From a purely mechanistic standpoint, given the relatively constant systemic levels of these (albeit) secondary charge carriers, one would have expected more consistent behaviour (as was in fact observed for lithium). A possible explanation lies in the fact that concentration gradients of calcium and potassium exist naturally in the epidermis. According to the literature (34,35), calcium levels increase significantly from the basal epidermis to the outer stratum granulosum (SG) and then decline precipitously in the stratum corneum, while potassium also increases up to a maximum just below the SG before falling off dramatically across the most superficial layers of the barrier. It is possible, therefore, that iontophoresis disturbs these ion concentration gradients (and presumably the intracellular/extracellular distribution of these ions as well) and provokes a subsequent attempt by the skin to re-establish the normal situation. Consequently, it is conceivable that a competitive and dynamic situation is induced as iontophoresis extracts these ions to the surface and the skin strives to maintain the *status quo*. Resolution of this point requires considerable further study.

When the applicability of Equation 3 was tested using the measured systemic levels of sodium, potassium and calcium, and their respective reverse iontophoretic flux ratios with lithium, only the lithium/sodium ratios gave acceptable results. Table I shows the linear regression results and clearly demonstrates that, after 30 minutes of iontophoresis, the ratio J_{Li}/J_{Na} should be usefully predictive of the serum lithium concentration ($r^2 > 0.92$). As observed for γ (the proportionality constant linking J_{Li} to C_{Li}), $\gamma^{\#}$, which links J_{Li}/J_{Na} to C_{Li}, becomes very stable after the first iontophoresis period, with a low error. In contrast, for potassium and calcium, the correlations between J_{Li}/J_K and C_{Li}, and J_{Li}/J_{Ca} and C_{Li}, were much less impressive: in the former case, r^2 at 90 and 120 minutes was 0.68 and 0.58, respectively; in the latter, the corresponding values were 0.80 and 0.64. It can be reasonably concluded, therefore, that sodium is by far the most suitable "internal standard" for "calibration-less" lithium monitoring.

D. Prediction of serum lithium concentration

In a final component of the investigation, the potential ability of the relationships derived to predict serum lithium levels from reverse iontophoretic extraction flux data was examined. Retrospectively, the patients were divided into two groups: The first ten patients acted as a "training set", the data from which were used to develop population values of γ and $\gamma^{\#}$ (specifically, from the regressions of J_{Li} and J_{Li}/J_{Na}, respectively, against C_{Li}). The results of this exercise are summarized in the upper half of Table II. With these proportionality constants, and the measured values of J_{Li} and J_{Li}/J_{Na} in the remaining patients (the "test group"), their C_{Li} were predicted and then compared to the actual, experimentally determined results. The outcome of this exercise is presented first in the lower half of Table II, which contains the results of simple linear regressions between the predicted and measured values of C_{Li} using the data from the two later periods of iontophoretic extraction. The correlations are excellent and the slopes closely approach the theoretical values of unity (especially for final interval of iontophoresis).

The approach was then better characterized by an inverse prediction procedure (25), which allowed the 95% confidence interval associated with each predicted value to be determined. Figure 5 compares the experimentally measured C_{Li} with the predicted results (together with the corresponding 95% confidence interval) based on the "training set" values of γ and $\gamma^{\#}$. In the former case, the *average* confidence intervals at 1.5 hours and 2 hours of iontophoresis were ±0.14 mM and ±0.19 mM, respectively. When $\gamma^{\#}$ was used, the corresponding values decreased to ±0.08 mM and ±0.11 mM. It is apparent that the predictability is rather good, with the iontophoretic extraction data at 1.5 hours providing systematically narrower confidence intervals, independent of whether γ

Table II: *Upper part:* Linear regressions, at different times of iontophoresis, of (i) the extracted lithium flux (J_{Li}) (data from Figure 3), and (ii) the ratio of extracted lithium and sodium fluxes (J_{Li}/J_{Na}), as a function of C_{Li}, according to Equations 2 and 3, respectively. Results are based on data from patients 1-10, the "training set". *Lower part:* Linear regressions between the predicted and measured values of C_{Li} in patients 11-23 (the "test group") using either J_{Li} or J_{Li}/J_{Na} and the values of γ and $\gamma^{\#}$ derived from the "training set" for the two later periods of iontophoretic extraction.

"Training set"

	J_{Li} versus C_{Li}			J_{Li}/J_{Na} versus C_{Li}		
Time (min)	$\gamma \pm$ SE (µl/h)	Intercept (nmol/h)	r^2	$\gamma^{\#} \pm$ SE (M^{-1})	Intercept	r^2
30	181 ± 69	-11	0.46	17.8 ± 5.4	$-3.\ 10^{-3}$	0.57
60	112 ± 22	0	0.77	7.7 ± 0.8	$-5.\ 10^{-4}$	0.92
90	114 ± 5	-12	0.87	7.1 ± 0.6	$-8.\ 10^{-4}$	0.95
120	90 ± 6	3	0.80	5.8 ± 0.7	$-3.\ 10^{-5}$	0.91

"Test group"

	Prediction from J_{Li} and γ			Prediction from J_{Li}/J_{Na} and $\gamma^{\#}$		
Time (min)	Slope ± SE	Intercept (µM)	r^2	Slope ± SE	Intercept (µM)	r^2
90	0.83 ± 0.07	93	0.93	0.82 ± 0.08	122	0.91
120	0.98 ± 0.09	-31	0.92	0.95 ± 0.08	23	0.93

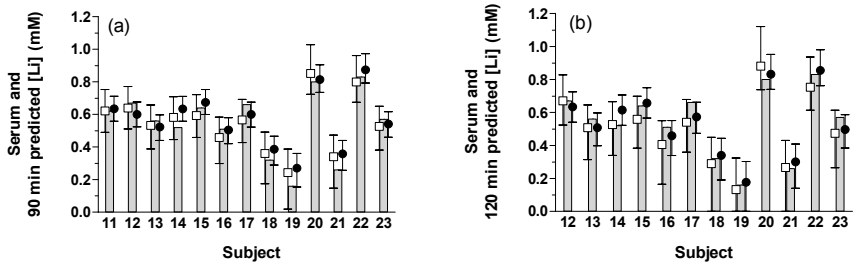

Figure 5: Comparison between measured C_{Li} (bars) in the "test group" of patients and the predictions (mean ± 95% confidence interval) based upon the values of γ (open squares) and $\gamma^{\#}_{Na}$ (filled circles) determined from the data of those patients (n=10) used as the "training set". Panel (a) shows the results based on the iontophoretic extraction fluxes (of Li^+ and Na^+) in the 60-90 minute interval (n=13), panel (b) uses data from the 90-120 interval (n=12).

or $\gamma^{\#}$ was used for the prediction. It is also important to point out that normalization of J_{Li} with the sodium extraction flux almost halved the average confidence interval associated with the prediction of C_{Li}, lending credibility and value to the "internal standard" calibration approach.

However, close inspection of Figure 5 shows that the confidence intervals for subjects 18, 19 and 21 are large. These patients had the lowest measured serum levels of lithium (0.16-0.32 mM), whereas the "training set" data fell in the range 0.42-0.79 mM. Statistically speaking, therefore, these three subjects fell outside the predictive capabilities of the model (25), and this emphasizes the importance of considerably expanding the database to ensure that the "training set" encompasses the widest possible range of C_{Li}. Nevertheless, it can be argued that this exercise has been generally successful and positive, and that a clear value for the "internal standard", in terms of improving data and in terms of predictive quality, has been demonstrated.

IV. Summary

This work shows that lithium can be easily extracted via transdermal iontophoresis in a concentration dependent manner. The variability of the extraction process is relatively low and has allowed (admittedly limited) predictive population extraction parameters to be estimated. The use of sodium as an "internal standard" decreases the error associated with the predicted serum values. The results support the premise, therefore, that transdermal iontophoresis constitutes an alternative to blood sampling for lithium monitoring *in vivo*. From a practical standpoint, considerable optimization of the approach is necessary, particularly with respect to maximizing extraction fluxes to shorten measurement times, and in terms of facilitating sample collection and analysis.

V. Acknowledgments and conflict of interest

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Drs Delgado-Charro and Guy are co-inventors of the PCT patent application (WO-A-03/000340): Method for non-invasively

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Chapitre 5

L'ionophorèse inversée du lithium : utilisation d'un gel polymérique thermoréversible pour la formulation des compartiments récepteurs

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Résumé

Buts : Cette étude propose une formulation gélifiée des chambres d'extraction par ionophorèse inversée. Facilitant son usage *in vivo* le gel doit être bon conducteur du courant et faciliter, autant que possible, l'étape analytique ultérieure. La formulation d'un gel polymérique (Pluronic F127) thermoréversible a été envisagée.

Méthodes : Les expériences de ionophorèse ont été menées in-vitro sur peaux dermatomisées d'oreille de porc. Le compartiment subdermique contenait une solution physiologique, du sodium en concentration fixe ainsi que du lithium en concentrations thérapeutiques variables. Un protocole de dialyse à l'équilibre a été mis au point dans le but d'extraire du gel les composés collectés par ionophorèse. Les quantités extraites de lithium et de sodium ont été analysées.

Résultats : Les résultats ont démontré *in vitro (a)* la faisabilité de l'extraction du lithium et du sodium dans un milieu d'extraction tamponné et gélifié avec du Pluronic F127 (20% m/m) et (b) qu'une technique simple de dialyse permet d'extraire les analytes du gel avant quantification.

Conclusions : Cette étude démontre l'utilisation possible d'un gel thermoréversible comme milieu d'extraction pour des applications d'ionophorèse inversée *in vivo*.

Mots-clés : Pluronic F-127, Polymère thermoréversible, Ionophorèse inversée, Lithium, Monitoring non-invasif

Reverse iontophoresis of lithium: electrode formulation using a thermoreversible polymer

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Keywords: Pluronic F-127, Thermoreversible polymer, Reverse iontophoresis, Lithium, Non-invasive monitoring

I. Introduction

Reverse iontophoresis has been proposed as an alternative procedure for non-invasively sampling drugs through the skin. Previous work *in vitro* and *in vivo* has shown that lithium, a frequently monitored drug, could be quickly and efficiently extracted via iontophoresis (1,2). Furthermore, this research has suggested that pharmacokinetic parameters of selected drugs, such as half-life, may be estimated from the iontophoretically extracted flux profiles. The use of sodium as an internal standard to normalize lithium iontophoretic extraction flux has also been investigated (1,2).

To date, simple aqueous solutions have been used to fill the electrode chambers on the skin surface into which the analyte of interest is extracted by iontophoresis. While this allows easy analytical chemistry, with little or no sample preparation, such vehicles are obviously not well adapted for practical purposes. In fact, the commercial development of iontophoretic drug delivery or sampling devices favours the use of gel or polymer formulations for the electrode compartments (3). A suitable vehicle for "*in vivo*" studies should combine a consistency that allows easy application and removal with appropriate conductive properties, as well as facilitating the subsequent analytical steps. It should be noted that an "off-line" assay of the drug is probably sufficient for drug monitoring and pharmacokinetic applications (i.e., an "on-board" biosensor as, for example, in the GlucoWatch Biographer® is not obligatory (4)).

The specific aim of this work was to formulate a gel or polymer-type collecting vehicle convenient for lithium reverse iontophoretic monitoring. As thermoreversible gels have been proposed as vehicles for iontophoretic drug delivery (5), it was decided to explore the possibility of using them as collection media for reverse iontophoresis. Pluronic F-127, a copolymer of polyoxyethylene and polyoxypropylene, (70/30 w/w; M.W. 11500), forms a gel at room temperature thereby allowing easy topical application and removal (5,6). Subsequently, on cooling to 4°C, the gel becomes fluid allowing the potential for facile extraction of the analyte(s) accumulated therein during reverse iontophoresis.

The first step was to formulate a conductive gel stable throughout the iontophoretic extraction period. Next, a dialysis procedure was developed to extract lithium and sodium from the collection gel. Finally, reverse iontophoresis of lithium (and sodium) was performed *in vitro* to compare the performance of the gel with that of a standard buffer solution.

II. Materials and Methods

Materials: 8 M LiCl solution was purchased from Fluka Chemie AG (Buchs, Switzerland). Pluronic F-127, Tris, Tris HCl, MOPS, NaCl, Ag wire 99,9%, AgCl 99%, Pt 99,9% were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France).

Gel formulation: Pluronic F-127 gel was prepared by the cold method (7,8). The required amount of Pluronic F 127 was dispersed in a cold pH 7.4 (Tris 32 mM, MOPS 34 mM) buffer. The solution was continuously stirred at 4°C until it was clear. For the dialysis experiments (see below) appropriate amounts of lithium and sodium chloride were added to the fluid preparation at 4°C.

Skin: Porcine ears were obtained fresh from the local slaughterhouse and were cleaned under running cold water. The tissue from the inner face of the ear was then dermatomed (Air Dermatome, Zimmer TM, France Medica, Illkirch, France) to a thickness of approximately 750 µm. The skin was either used at once or stored at –20°C for no longer than one month before use.

Reverse iontophoresis experiments: The skin was clamped between the two halves of vertical Franz diffusion cells (2,63 cm^2). The lower half was kept at 37°C in a water bath and represented the anodal subdermal compartment, which was filled with a pH 7.4 buffer (133 mM NaCl, 32 mM Tris, 34 mM MOPS). LiCl was added to this buffer at one of three concentrations (0.5; 0.9; and 1.4 mEq/l) spanning the therapeutic range of the drug. The upper half of the cell acted as the cathodal collecting chamber and was filled with 3 ml of either pH 7.4 buffer (32 mM Tris, 34 mM MOPS) or 20 % (w/w) Pluronic F-127 in the same buffer. The iontophoretic extraction consisted of a single 90 minutes application of direct constant current (1 mA) delivered via Ag/AgCl electrodes. At the end of the iontophoretic procedure, the cells were placed for exactly 6 minutes at -20°C to provoke the vehicle transition into the fluid phase. This time was fixed in order to ensure the same contribution of passive diffusion to the total transport in all replicates. The fluid receiver solutions were then homogenized and removed for analysis.

Dialysis: A dialysis method was used to recover sodium and lithium from the Pluronic F-127 vehicles. Side-by-side Teflon cells (1 ml compartment volume and 0.79 cm^2 dialysis surface) were employed. A cellulose dialysis membrane (Spectra/Por® 6, MWCO 2000, Spectrum® Laboratories, Inc., Rancho Dominguez, California, USA) was clamped between the two compartments with the assistance of two silicone rings. 1 ml of a fluid Pluronic receptor solution and 1 ml of a pH 7.4 buffer solution (32 mM Tris and 34 mM MOPS) were placed into the donor and receiver compartments, respectively. The ensemble was kept at 4°C and continuously stirred for 12 hours. Other dialysis times were also studied (3, 6 and 24 hours).

Assay: Lithium and sodium analysis were quantified by ion chromatography. A Dionex DX-600 (Dionex, Voisins le Bretonneux, France) equipped with a GP-50 pump (1 ml/min), a AS-50 thermal compartment (25°C), a ED-50 detector (61 mA), a CS-16 column and a 6 mM H_2SO_4 mobile phase were used. Peak integration was performed with a Chromeleon TM v.6.4. software. Retention times of lithium and sodium were 6.8 and 11.8 minutes, respectively.

Statistics: Linear regressions were performed using Software Prism 4 (GraphPad Software, San Diego, CA). All regressions shown in this work were significant ($P<0.01$). Data are presented as the mean and standard deviation of at least 4 replicates.

III. Results and discussion

The effectiveness of the 20% w/w Pluronic F-127 buffer solution to act as an iontophoretic vehicle was first investigated. As well as a sufficient electrical conductivity, the formulation must permit the appropriate electrode reactions to proceed throughout the period of iontophoresis. Silver is reduced at the cathode, a reaction which requires only that the AgCl coating of the electrode is sufficient. Thus, the electrolytes added to the Pluronic solution (Tris and MOPS) served only for buffering and conductivity purposes. In contrast, silver oxidation occurs at the anode and the formulation must therefore provide enough chloride ions to avoid alternative electrochemistry and the precipitation of insoluble silver hydroxy species (9). It was found that 200 mM NaCl had to be added to the gel formulation to create a useable anodal vehicle. This rather high level was necessary because the slow diffusion kinetics in the gel meant that only the chloride ions relatively close to the anode were "available" to participate in the electrode reaction. To avoid significant depletion, therefore, it was prudent to provide a high chloride content in the gel.

Overall, the Pluronic F-127 solution worked very efficiently as a cathodal formulation into which lithium and sodium

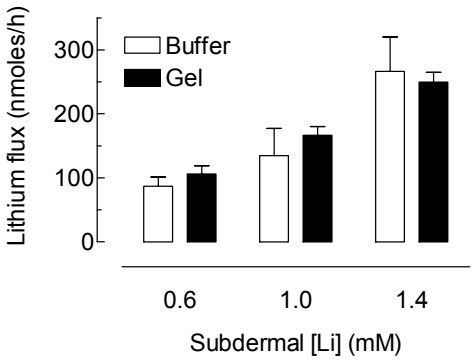

Figure 1: Reverse iontophoretic extraction fluxes of lithium into either an aqueous or a gel collection medium. The data represent the mean ± SD of 4 replicates.

(and, presumably, other cationic, neutral and zwitterionic analytes) could be extracted. Its use as an anodal formulation, on the other hand, would require either a careful titration of the chloride concentration or the use of a salt bridge.

Ion chromatography requires aqueous samples and it was therefore necessary to perform a simple dialysis procedure to separate the two cations from the polymeric vehicle. First of all, the linearity of the extraction process was verified. Reference gels were prepared to which different concentrations of lithium (20-100 µM) and sodium (2-10 mM) had been added. The ranges of concentrations were based upon the iontophoretic extraction efficiencies observed for the cations in previous work (1,2). After dialysis for 12 hours, the cationic content of the aqueous solution was quantified. Both sodium and lithium extractions were linear ($r^2 > 0.99$ for both cations). This simple procedure could be used, therefore, to process the samples. While other dialysis times (3, 6 and 24 hours) yielded equally good results, a 12 hour duration was preferred for practical reasons. The principal limitation of the approach was dilution of the analytes by the dialysis, a possibly critical

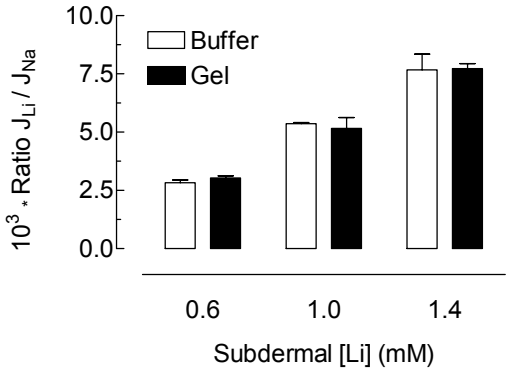

Figure 2: Ratio of lithium to sodium extraction fluxes into either an aqueous or a gel collection medium. The data represent the mean ± SD of 4 replicates.

Table I: Linear regressions of the data shown in Figures 1 and 2 according to the equation: Y = K . Concentration + Intercept, where Y is either the lithium extraction flux in nmol.h-1, or the ratio (x 10^3) of fluxes J_{Li}/J_{Na}, and Concentration refers to the subdermal level of the drug in mM.

Y	Formulation	K	Intercept	r^2
Lithium flux	Gel	161 ± 12 [a]	12 ± 13	0.94
	Buffer	200 ± 36 [a]	-38 ± 39	0.75
10^3 . J_{Li}/J_{Na}	Gel	5.3 ± 0.3 [b]	-3.10^{-3} ± 0.3	0.97
	Buffer	5.5 ± 0.3 [b]	-0.2 ± 0.3	0.97

[a] Units are (flux) /(Concentration)
[b] Units are $(Concentration)^{-1}$

issue for substances less efficiently extracted than lithium and sodium (in which case a method allowing direct quantification in the gel would be desirable).

Reverse iontophoretic extraction of lithium and sodium demonstrated the performance of the gel as a cathodal receptor media. Control experiments used the same buffer but without the polymer. Figures 1 and 2 show the lithium extraction fluxes and the extraction flux ratio, J_{Li}/J_{Na}, for the gel and solution formulations as a function of the subdermal lithium concentration. A 2-way ANOVA demonstrated, for both J_{Li} and J_{Li}/J_{Na} a significant dependence on concentration ($p<0.001$). There was no significant difference, however, between the extraction fluxes of either lithium or sodium into the gel and the solution. Linear regressions between both J_{Li} and J_{Li}/J_{Na} and the subdermal concentration of the drug are shown in Table I. The results are very similar and no statistical differences were found between the slopes and Y-intercepts in any case. This shows that the F-127 gels can be successfully employed as vehicles in reverse iontophoresis and that the analytes were proportionally extracted by equilibrium dialysis before quantification by ion chromatography. It was also noted that the use of sodium as an internal standard resulted in reduced variability, suggesting that some of the experimental error had been eliminated by this procedure.

In summary, this study demonstrates the potential value of thermoreversible gel formulations as collection media for reverse iontophoresis applications. A simple technique, such equilibrium dialysis, can (if necessary) be used to separate the compounds of interest from the polymer to facilitate the subsequent analytical procedures.

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Conclusions et perspectives

Dans son ensemble, ce travail met en évidence le potentiel de l'ionophorèse inversée comme méthode alternative au prélèvement sanguin dans un contexte de surveillance thérapeutique. Le choix judicieux des deux molécules modèles, phénytoïne et lithium, a permis d'illustrer les capacités et les bénéfices de cet outil de diagnostique non-invasif.

Aptitude de l'ionophorèse à réaliser un monitoring thérapeutique

Quelle que soit l'ionisation de la molécule (cationique pour le cas du lithium, anionique et non-ionisée pour la phénytoïne), et le mécanisme d'extraction (électromigration ou électroosmose) de bonnes corrélations ont été obtenues entre les concentrations subdermiques et les quantités extraites par ionophorèse. Cette étude confirme aussi que le meilleur candidat à l'extraction ionophorètique est de petite taille. De plus pour des raisons analytiques de quantification dans les extraits, les meilleurs candidats sont des médicaments en concentrations thérapeutiques importantes dans la circulation systémique et si possible bien ionisés de façon à profiter du mécanisme d'extraction transdermique le plus efficace qui soit : l'électromigration.

Des différences notables ont été observées entre ces deux principes actifs. Par exemple, les délais nécessaires à la stabilisation de l'extraction sont très différents : 6h pour le lithium et 15h pour la phénytoïne. Ces résultats sont notamment dûs aux différences physicochimiques des deux médicaments. Sans doute la taille et la lipophilie de la phénytoïne sont telles qu'elles deviennent des facteurs déterminant dans la lente stabilisation du flux d'extraction ionophorétique. Cependant, des corrélations satisfaisantes ($r^2 \geq 0.80$) ont été obtenues après 1h30 pour le lithium, et 4 à 6h pour la phénytoïne, ce qui laisse envisager des temps d'application plus courts. Mais seules des études complémentaires *in vitro* puis *in vivo* pourront permettre de confirmer ces résultats.

Cela a été le cas pour le lithium. L'étude préliminaire réalisée *in vivo* sur 23 patients confirme que l'extraction du lithium est bien linéairement dépendante de la lithiémie sérique ($r^2 > 0.9$) après 60 minutes d'extraction. Cette étude souligne aussi la faible variation inter-individuelle de l'extraction ionophorètique du lithium. Ces résultats laissent envisager le développement d'un système ambulatoire complètement non-invasif par voie transdermique nécessitant, au préalable, une étape d'optimisation opérationnelle et analytique.

Mesure des concentrations libres de principes actifs par ionophorèse

Il a été montré que seule la fraction libre de la phénytoïne est extraite, le complexe phénytoïne/albumine étant trop imposant pour traverser les barrières successives de la peau. L'ionophorèse inversée a démontré ses capacités à suivre les modifications de la fixation protéique (par exemple : une hypoalbuminémie, une interaction médicamenteuse, …). Ceci souligne l'intérêt particulier de la technique pour des médicaments dont la fixation est dépendante de la concentration thérapeutique (par exemple : acide valproique).

Aptitude de l'ionophorèse à suivre des profils pharmacocinétiques

Pour un médicament aussi mobile que le lithium, les flux d'extraction ionophorètiques suivent rapidement les changements apportés aux concentrations subdermiques. Pour des médicaments dont les propriétés sont proches de celles du lithium, l'ionophorèse démontre, *in vitro*, un fort potentiel pour la réalisation d'études pharmacocinétiques. L'accès à la constante d'élimination (ainsi qu'à la demi-vie) de manière complètement non-invasive constitue un atout important de cette technique. De plus, dès lors que les constantes d'extraction ionophorètiques sont connues, la technique permet l'accès aux autres paramètres. Nécessairement, d'autres études *in vitro* et *in vivo* devront confirmer ces résultats encourageants pour la réalisation d'études pharmacocinétiques aussi peu invasives que possible.

Calibration pour rendre la technique complètement non invasive

Dans un contexte de monitoring thérapeutique, ne nécessitant classiquement qu'une seule prise de sang, la méthode ne serait d'aucune utilité clinique si elle nécessitait une calibration invasive. Les résultats in vivo de monitoring transdermique montrent que l'extraction du lithium est efficace et peu sensible aux variations inter-individuelles laissant envisager qu'une calibration n'est pas nécessaire. Néanmoins, une calibration par le sodium a permis de réduire sensiblement l'erreur sur les valeurs prédites de lithiémie. Ces résultats préliminaires restent à être confirmés sur une population plus vaste. Pour beaucoup d'autres médicaments moins mobiles, la calibration pourrait s'avérer indispensable. Cette étude démontre, *in vitro* et *in vivo*, la validité du concept de calibration par un « standard interne », rendant la technique complètement non-invasive. Son utilisation toujours satisfaisante, place le sodium comme un candidat de choix. Néanmoins, le choix d'un standard interne restera un challenge pour chaque couple médicament/standard interne. Des études approfondies devront estimer la variabilité de l'extraction du standard interne et son insensibilité vis-à-vis des changements de concentrations subdermiques du médicament. Inévitablement, l'optimisation de cette approche nécessitant la quantification des flux d'extraction des deux analytes passera par un développement analytique plus complexe.

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Leboulanger B., Guy R.H., Delgado-Charro M.B. Reverse iontophoresis for non-invasive transdermal monitoring. *Physiol Meas.* 2004 Jun; 25(3):R35-50

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