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Research paper

# Extraction of amino acids by reverse iontophoresis *in vivo*

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ABSTRACT

Reverse iontophoresis across the skin is a potentially useful alternative for non-invasive clinical and therapeutic drug monitoring. In this work, the reverse iontophoretic extraction of 17 amino acids was studied *in vivo* in healthy volunteers. Charged amino acids were primarily extracted towards the electrode of opposite polarity, while zwitterionic species were extracted, more or less equally, to both anode and cathode, suggesting that the net charge on the skin, under the conditions of the experiment, was close to zero. The significant presence of a 'skin reservoir' of several amino acids, presumably originating from the barrier's so-called 'natural moisturising factor', was deduced from the results. While this phenomenon had been observed in an earlier *in vitro* investigation, the levels of certain amino acids (including serine and glycine) in the skin were found to be much higher *in vivo*. Hence, while the results of this study confirm the feasibility of extracting some amino acids at physiologically relevant levels *in vivo*, the objective of achieving a correlation between iontophoretically extracted fluxes and blood plasma levels may not be a practically realisable goal in all cases.



## 1. Introduction

Over the past two decades, reverse iontophoresis across the skin has been investigated as an alternative, non-invasive method for clinical and therapeutic drug monitoring [1]. Its first application, the transdermal extraction of glucose [2], led to the development of the GlucoWatch® Biographer (Johnson & Johnson, New Brunswick, NJ). This device, which has been approved by the U.S. Food and Drug Administration, uses reverse iontophoresis to monitor glycaemia transdermally for up to 12 h [3]. More recent work has confirmed the potential of the approach to track (both *in vitro* and *in vivo*) subdermal levels of lithium [4], phenytoin [5], valproate [6], lactate [7], and urea [8]).

Amino acids are biological markers, the plasma levels of which may be used to detect inherited metabolic diseases [9]. Because of their small molecular weight and polar nature, these compounds are excellent candidates for non-invasive monitoring via reverse iontophoresis. Indeed, previous work has shown how the transport of amino acids across the skin can be significantly enhanced by iontophoresis [10,11], and pilot investigations (again, both *in vitro* and *in vivo*) on the reverse iontophoretic extraction of phenylalanine for the diagnosis of phenylketonuria have been reported [12,13].

Iontophoresis uses a small electric current to drive charged and highly polar compounds across the skin at rates very much greater than their passive permeabilities [1]. Two major transport mechanisms are involved: electromigration and electroosmosis. Electromigration is the movement of small ions across the skin under the direct influence of the electric field. Electron fluxes are transformed into ionic fluxes via the electrode reactions, and ionic transport proceeds through the skin to maintain electroneutrality.

The total charge transported depends on the strength of the electric field and the duration of application. Iontophoresis sets in motion a number of ions across the skin, and all of them compete to carry a fraction of the current. According to Faraday's law, the flux of each ion in the iontophoretic circuit is given by

$$J_i = (t_i * I)/(F * z_i) \quad (1)$$

where $J_i$ is the flux of the $i$th ion, $t_i$ is its transport number, $z_i$ is the valence, F is Faraday's constant and $I$ is the total current flowing. Iontophoretic transport numbers depend on the relative mobility and concentration of all mobile ions in the system [14].

Electroosmosis is the principal transport mechanism of uncharged molecules and high molecular weight cations. The skin is negatively charged at physiological pH [15] and acts, therefore,

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as a permselective membrane to cations. This preferential passage of counterions induces an electroosmotic solvent flow that carries neutral molecules in the anode-to-cathode direction. The volume flow $J_V$ (volume per unit time per unit area) is predicted [16] to be proportional to the potential gradient established by the electric field. The molar flux of a solute "j" present at concentration $c_j$ is then represented as

$$J_j = J_V * c_j \quad (2)$$

Electroosmosis depends on the charge on the membrane and may be modified by the formulations (e.g., the pH) which "couple" the electrodes to the skin. Typically, for analytes such as amino acids, both transport mechanisms contribute to the iontophoretic extraction, the fraction of electromigration and electroosmosis depending on the physicochemical properties of the analyte.

Many diverse metabolic diseases are characterized by pathological, systemic levels of at least one amino acid [9]. Plasma concentrations can therefore be used to detect the disease and to monitor the success of therapeutic measures, such as a strict diet. The advantage of reverse iontophoresis as a monitoring tool is the non-invasive nature of the technique, and the possibility to follow simultaneously a variety of analytes – for example, several amino acids – in the same skin extract. However, due to the skin's impressive barrier function, a transdermal extract contains a much lower concentration of the analyte than that present in the blood. A significant challenge, therefore, is to detect and quantify reproducibly the analyte of interest over the concentration range that is clinically relevant. The feasibility of achieving this objective has been recently demonstrated *in vitro* [17].

As the next step, the research presented in this paper examined whether reverse iontophoresis, together with sample analysis using liquid chromatography coupled with mass spectrometry (LC–MS), could permit the simultaneous detection of 17 naturally occurring amino acids *in vivo*. The second goal was to determine if there is a relation between iontophoretically extracted fluxes and the corresponding blood plasma levels. The analytes were measured, subsequent to current passage, in both the anode and cathode chambers, permitting evaluation of the controlling structure-transport relationships in operation.

## 2. Materials and methods

### 2.1. Study population

Six healthy subjects (aged 20–51 years; three males, three females), with no history of skin disease participated in the study. Informed consent was obtained, the study protocol having been approved by the internal review board of the University Hospital of the Canton of Geneva in accord with the principles outlined in the Declaration of Helsinki. The subjects fasted throughout the experiment, but were allowed free access to water.

### 2.2. Chemicals

All chemicals were purchased from Sigma Aldrich (St. Quentin Fallavier, France). The purchased amino acids were purchased as two standard solutions, one for neutral and acidic amino acids (β-alanine, L-alanine, L-α-aminoadipic acid, L-*a*-amino-*n*-butyric acid, D,L-α-aminoisobutyric acid, L-asparagine, L-aspartic acid, L-citrulline, cystathionine, L-cystine, L-glutamic acid, glycine, hydroxy-L-proline, L-isoleucine, L-leucine, L-methionine, L-phenyalanine, *O*-phospho-L-serine, *O*-phosphoethanolamine, L-proline, L-sarcosine, L-serine, taurine, L-threonine, L-tyrosine, urea and L-valine; 2.5 mM in 0.1 M HCl), and the other for basic amino acids (γ-amino-*n*-butyric acid, ammonium chloride, L-anserine, L-arginine, L-carnosine, ethanolamine, L-histidine, L-homocysteine, D-hydroxylysine, L-lysine, 1-methyl-L-histidine, 3-methyl-L-histidine, L-ornithine; 2.5 mM in 0.1 M HCl). Acetonitrile and nonafluoropentanoic acid and two deuterated internal standards were of analytical grade, and sodium chloride was acquired as 154 mM physiological solution. All solutions were prepared with bi-distilled water (resistivity >18 MΩ/cm$^2$).

### 2.3. Iontophoresis

Two cylindrical glass cells (diameter 1.6 cm, extraction surface 2 cm$^2$), separated by a distance of 7 cm, were fixed with foam tape (3 M, Health Care) to the ventral forearm. Both chambers were filled with 0.8 ml of unbuffered 30 mM NaCl solution. Custom-made Ag/AgCl electrodes were inserted into the solutions and fixed above the skin surface to ensure that no physical contact with the skin occurred. Direct current ($I$ = 0.6 mA, current density = 0.3 mA/cm$^2$) was passed for a total of 4 h and was controlled by a Phoresor II Auto (Iomed, Salt Lake City, UT), an FDA-approved, constant current, iontophoretic power supply. Every 25 min post-initiation of the current, the entire cathodal and anodal solutions were collected and replaced with 0.8 ml of fresh NaCl solution. After the last collection interval, an intravenous blood sample was obtained from which the plasma was separated by centrifugation. The iontophoresis and plasma samples were immediately frozen and stored at −80 °C until analysis.

### 2.4. Analytical chemistry – liquid chromatography coupled to mass spectrometry (LC–MS)

The identification and simultaneous quantification of the 17 amino acids were based on a previously published method [18,19]. LC–MS was performed on a HP Series 1100 LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a binary pump. The Chemstation suite software (Agilent Technologies) was used for instrument control, data acquisition and data handling. Detection was carried out with a HP Series 1100 MSD (Agilent Technologies) equipped with an orthogonal electrospray interface. Nitrogen was used as both a nebulising (35 psi) and a drying gas at a temperature of 350 °C at a flow rate of 12 L min$^{-1}$. Electrospray and skimmer voltages were set at 1000 and 90 V, respectively. The quadrupole was operated in the selected ion monitoring (SIM) mode and was set at 19 ions. Amino acids are separated into four groups depending on retention time to reach a minimum dwell time of 50 ms for each ion. The protonated molecule $[M+H]^+$ was used as the selected ion. m/z are for group 1 from 5.1 to 15.9 min (Asn 133, Gly 76, Ser 106, Asp 134, Gln 147, Glu 148, Thr 120, Ala 90), group 2 from 16 to 19.7 min (Pro 116), group 3 from 19.8 to 23.2 min (Val 118, Tyr-D4 186, Tyr 182, Met 150) and group 4 from 23.4 to 29.5 min (Ile 132, Leu 132, His 156, Lys 147, Phe-D5 171, Phe 166, Arg 175).

The chromatographic separation was performed on a Symmetry C18, 3.5 μm, 2 mm i.d. × 150 mm (Waters, Switzerland) fitted with a Symmetry C18, 3.5 μm, 2 mm i.d. × 5 mm, (Waters) pre-column. The separation was carried out at a flow rate of 200 μl min$^{-1}$ with the following solvent system: (A) a 20 mM nonafluoropentanoic acid solution and (B) acetonitrile. Ten microliters of internal standard solution (10 μM of Phe-D5 and Tyr-D4 in 30 mM NaCl) were added to 90 μl of each iontophoretic sample without any other pretreatment. The samples were vortexed, centrifuged and transferred to HPLC-vials. The injection volume was 20 μl.

The gradient elution program started with 99:1 (v/v) A:B for 5 min, followed by a linear change to 82:18 (v/v) A:B over 7 min, then altered linearly again to 74:26 (v/v) A:B over 6 min, and subsequently to 50:50 (v/v) A:B over another 6 min, and finally with an isocratic elution at 50:50 (v/v) A:B for 5 min. The analytical col-

umn was washed with 20:80 (v/v) A:B for 3 min at a flow rate of 300 μl $min^{-1}$. The system was equilibrated for 16 min under the initial conditions. Analyses were carried out at 10 °C.

This analytical method was fully validated to detect the amino acids in the iontophoretic matrix [20] according to ICH guidelines, and detailed results will be presented elsewhere. Briefly, trueness, repeatability (within-day variability) and intermediate fidelity (between-day variability) were evaluated for three series (independent analysis days) with two calibration curves (three concentration levels, 0.1, 2.5 and 10 μM). Each day, four concentrations (0.1, 0.25, 1, 2.5 and 4 μM) were evaluated in triplicate with three batches of internal controls (validations standards) and with two batches of external controls (0.25 and 2.5 μM). For calibration, a solution of 30 mM NaCl was used to mimic the composition used in the iontophoretic cells. For all the tested levels, trueness, repeatability and intermediate fidelity were between 5% and 15%. The working range for Glu, Thr, Ala, Pro, Val, Tyr, Met, Ile, Leu, His, Phe, Arg, Asp, and Gln was 0.1–10 μM; for Gly and Lys, 1–10 μM; and for Ser, 2.5–10 μM. The lower limits of these ranges correspond to the limit of quantification (LOQ). It has to be noted that the high concentration of salts in the iontophoretic samples (30 mM NaCl and the other extracted salts) produced signal suppression in the first part of the chromatogram. Results for amino acids detected in this analysis time window (Asn, Ser, Gly, Asp and Gln) were slightly higher with CVs ⩽25%. This value was considered acceptable according to the important variation between extractions due to the biological variability. Asn was the most affected by signal suppression and could not be quantified at the concentration levels required for this series of experiments.

The plasma samples were analysed for amino acids via a routine ion-exchange chromatography method using a Beckman analyser (Beckman Coulter, Nyon, Switzerland) [21].

### 2.5. Data analysis and statistics

Iontophoretic fluxes of all amino acids were directly calculated from the amount (mol) extracted in each collection interval normalised for the duration of the interval (expressed in h) and extraction surface ($cm^2$). For the analysis of transport direction and efficiency of extraction, the 4-h, apparent steady-state fluxes were considered. To examine the relationship between flux and plasma levels, the amino acid fluxes were normalised by the corresponding plasma concentration.

Where appropriate, data were expressed as mean ± standard deviation (SD). Statistical tests were performed with Graph Pad Prism 4.00 software (San Diego, CA).

## 3. Results and discussion

All subjects experienced a mild tingling sensation when the current was applied. Generally, the sensation diminished with time of current application and lasted no longer than 30 min. Iontophoresis caused the skin sites beneath the electrode chambers to become slightly erythematous, an effect which disappeared within 24 h.

Following initiation of iontophoresis, the amino acid fluxes decreased over time before stabilising in most cases after 2–3 h of current passage (data not shown). This suggests the presence of a skin reservoir for the amino acids as has been reported for a number of other analytes including glucose, urea, and lactate [3,7,8]. From the practical standpoint of monitoring systemic amino acid levels, this observation means that a pre-iontophoresis period would be necessary to deplete the analyte in the skin before the extraction flux could mirror accurately the subdermal concentration.

The steady-state extraction fluxes of the amino acids were determined from the amount in the last collection interval, that is, after 4 h of iontophoresis. Fig. 1 shows these fluxes for each subject; the average values resulting from all subjects are given in Table 1. In general, the amino acids were extracted in the nanomolar range, with significant variation between subjects. The cationic (Arg, Lys and His) and anionic (Asp and Glu) amino acids were predominantly extracted towards the chamber of opposite charge, although low amounts of these species were also found in the other electrode chamber. For the zwitterionic amino acids, the situation was less clear-cut. In all subjects, Ser and Gly showed the highest extraction levels, with extraction predominantly towards the anode. For the other zwitterions, the extraction was sometimes greater towards the anode (P1, P2), sometimes to the cathode (P4, P5), and sometimes showed no difference (P3, P6).

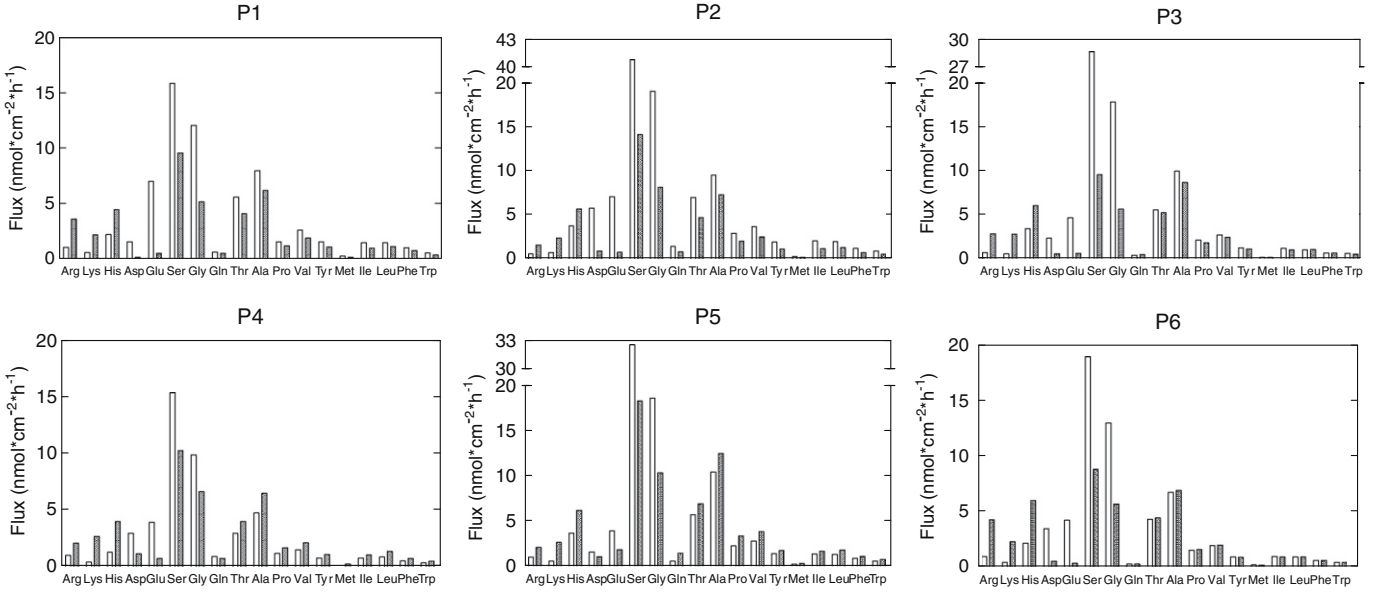


**Fig. 1.** Reverse iontophoretic extraction fluxes for each amino acid in each subject after 4 h of iontophoresis. The open bars represent extraction towards the anode, shaded bars towards the cathode.

**Table 1**
Extraction fluxes of amino acids after 4 h of iontophoresis to the anode and cathode, the corresponding plasma concentrations, the normalised flux values (absolute flux divided by plasma concentration), and the cathode/anode flux ratios.

| AA | pI | Anodal flux (nmol $h^{-1}$) | Cathodal flux (nmol $h^{-1}$) | Plasma μM) | Normalised anodal flux (μl $h^{-1}$) | Normalised cathodal flux (μl $h^{-1}$) | Flux ratio cathode/anode |
|---|---|---|---|---|---|---|---|
| Arg | 10.76 | 1.4 ± 0.6 | 5.0 ± 2.4 | 81 ± 25 | 21 ± 6 | 70 ± 13 | 3.57 |
| Lys | 9.47 | 1.0 ± 0.2 | 5.0 ± 0.4 | 177 ± 20 | 6 ± 2 | 28 ± 4 | 5.00 |
| His | 7.64 | 5.4 ± 2.0 | 10.8 ± 1.8 | 89 ± 11 | 62 ± 25 | 122 ± 25 | 2.00 |
| Glu | 3.08 | 10.2 ± 3.0 | 1.6 ± 1.0 | 20 ± 4 | 526 ± 202 | 78 ± 50 | 0.16 |
| Asp | 2.98 | 5.8 ± 3.2 | 1.4 ± 0.8 | 3 ± 0 | 1880 ± 1240 | 442 ± 276 | 0.24 |
| Thr | 5.87 | 10.4 ± 2.8 | 9.8 ± 2.2 | 120 ± 13 | 86 ± 23 | 82 ± 20 | 0.94 |
| Ala | 6.11 | 16.4 ± 4.4 | 16.0 ± 4.6 | 272 ± 46 | 62 ± 16 | 58 ± 14 | 0.98 |
| Pro | 6.30 | 3.8 ± 1.2 | 3.8 ± 1.6 | 155 ± 53 | 28 ± 18 | 26 ± 10 | 1.00 |
| Tyr | 5.63 | 2.6 ± 0.8 | 2.2 ± 0.6 | 189 ± 20 | 14 ± 4 | 12 ± 3 | 0.85 |
| Met | 5.74 | 0.4 ± 0.2 | 0.4 ± 0.2 | 22 ± 3 | 12 ± 8 | 14 ± 5 | 1.00 |
| Ile | 6.04 | 2.4 ± 1.0 | 2.2 ± 0.6 | 55 ± 8 | 45 ± 14 | 40 ± 10 | 0.92 |
| Leu | 6.04 | 2.4 ± 0.8 | 2.4 ± 0.6 | 109 ± 13 | 22 ± 8 | 22 ± 6 | 1.00 |
| Phe | 5.91 | 1.6 ± 0.6 | 1.4 ± 0.4 | 48 ± 6 | 32 ± 12 | 30 ± 5 | 0.88 |
| Gln | 5.65 | 1.2 ± 0.8 | 1.4 ± 0.8 | 549 ± 99 | 2 ± 2 | 2 ± 2 | 1.17 |
| Val | 6.00 | 5.0 ± 1.6 | 4.8 ± 1.4 | 45 ± 9 | 116 ± 50 | 110 ± 28 | 0.96 |
| Gly | 6.06 | 30.0 ± 7.8 | 13.8 ± 4.0 | 178 ± 54 | 186 ± 92 | 84 ± 38 | 0.46 |
| Ser | 5.68 | 50.6 ± 20.6 | 23.4 ± 7.4 | 102 ± 14 | 516 ± 260 | 234 ± 85 | 0.46 |

For clinical monitoring, a clear correlation between the iontophoretically extracted flux and the blood plasma value is required for each analyte of interest. The ranges of measured plasma concentrations of the amino acids are compared in Fig. 2 to the reference values in the literature [9] and, generally speaking, a reasonable overlap was found. It is then convenient to normalize the extracted fluxes by the plasma concentration to correct for variations in the iontophoretic flux that are due to different plasma levels.

Inspection of the extraction fluxes in Table 1 reveals, as mentioned above, the anticipated, enhanced transport of cationic amino acids to the cathode and anionic amino acids to the anode. Fig. 3(a and b) illustrate this point graphically for histidine and glutamic acid, showing the results both in terms of the absolute fluxes measured and as ratios of these fluxes to the corresponding plasma concentrations. For the zwitterionic amino acids, on the other hand, it is difficult to discern any obvious relationship between the extraction flux to either the anode or the cathode and the corresponding plasma concentration. Fig. 3(c) demonstrates this point for alanine. Given that one might expect the predominant mechanism of electrotransport, in this case, to be electroosmosis, it would follow that normalisation of the flux by the corresponding plasma level should result in a series of relatively constant values for the resulting ratio (in line with Eq. (2)). However, as indicated in the last two columns of Table 1, this turns out not to be the case, and normalised fluxes ranging from 2 to 250 μl/h are observed.

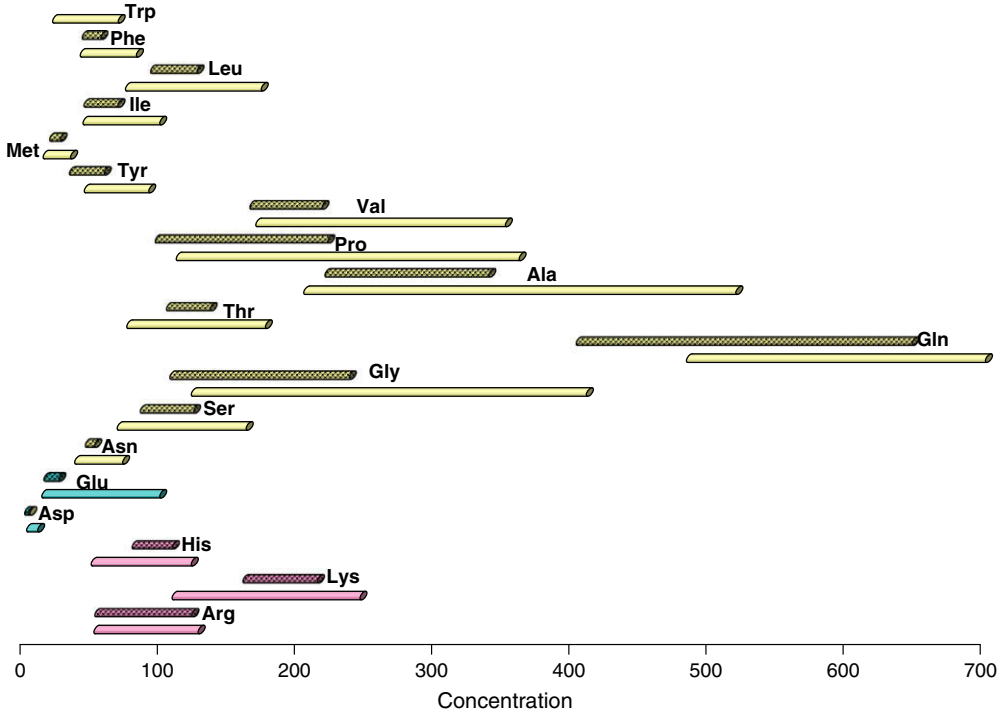


**Fig. 2.** Reference amino acid plasma values (open bars) and the ranges of concentrations observed in this study (shaded bars). All concentrations are given in μM.

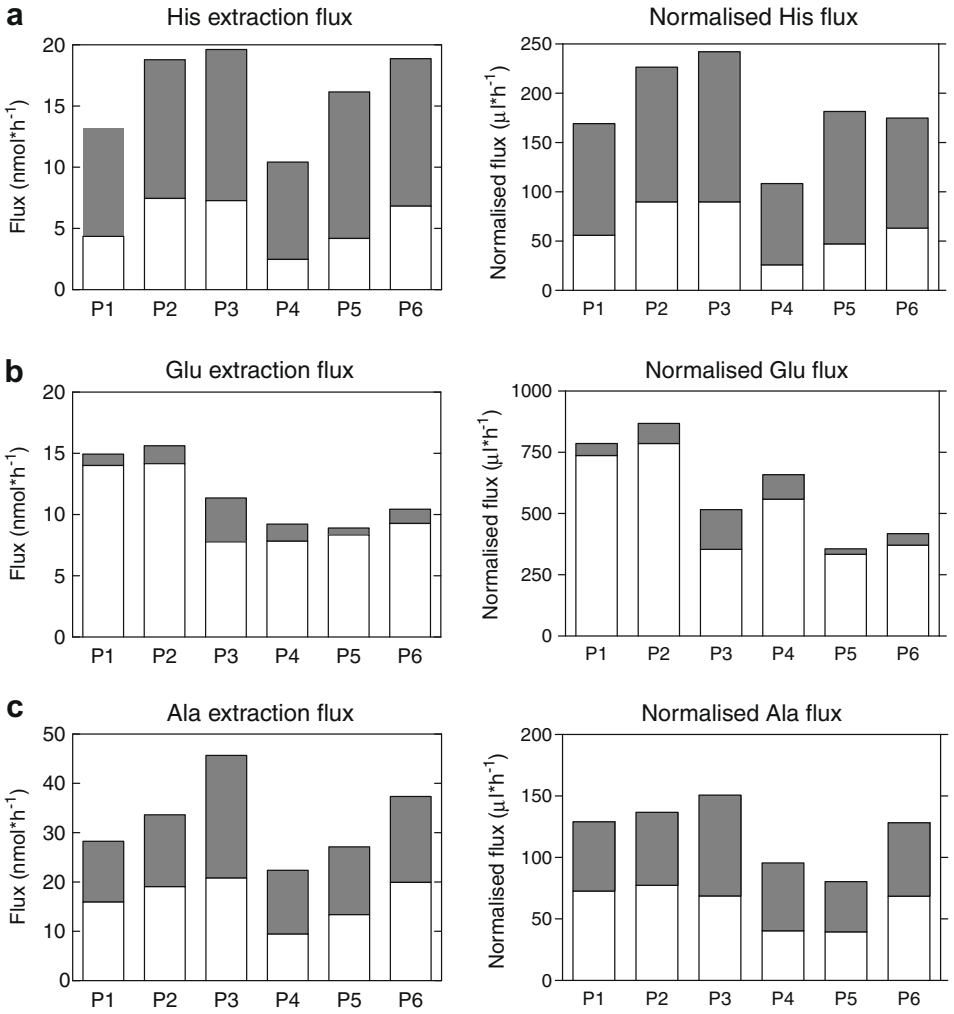


**Fig. 3.** Extraction fluxes of selected amino acids [(a) histidine, a cation, (b) glutamic acid, an anion, and (c) alanine, a zwitterion] after 4 h of iontophoresis to the anode (open bars) and cathode (shaded bars), and the corresponding normalised flux values (absolute flux divided by plasma concentration).

It was noted before that the initial period of iontophoresis yields higher extracted amounts of the amino acids (relative to the 'steady-state' values after 4 h of current passage), and this has been interpreted in terms of a "reservoir" for these analytes in the skin [17]. Given the magnitude of the normalised fluxes determined from the 4-h extraction data for some of the amino acids (such as serine, glycine, valine, threonine and alanine), and the fact that not all of these compounds are present in plasma at high concentration, it may be deduced that the "skin reservoir" for these species is considerable, and that the use of reverse iontophoresis to monitor fluctuations in their systemic levels is unlikely to be feasible. The contribution of various amino acids to the skin's natural moisturising factor (or NMF) undoubtedly explains the significant presence of these moieties in the *in vivo* barrier [22].

Comparison of the results from this human volunteer study with those obtained previously from *in vitro* experiments using excised porcine skin [17] is instructive (Fig. 4). In the earlier work, the amino acid concentrations in the subdermal compartment (the *in vitro* equivalent of the plasma, in effect) were precisely controlled at specific levels, which were not always relevant to those observed in real life. The iontophoretic current used *in vitro* also differed from that employed in the present study. Nevertheless, a direct comparison between the extraction fluxes is possible once the values have been normalised by the appropriate driving concentration (i.e., that subdermally *in vitro* and that in the plasma *in vivo)* and by the applied current. Qualitatively, there is a general agreement: the charged amino acids transport preferentially in the expected direction, and the zwitterions show only small differences in their levels of extraction to anode and cathode. Quantitatively, though, there is divergence in the *in vitro* and *in vivo* behaviours. For the charged amino acids, for example, there was a measurable extraction to the "wrong" compartment *in vivo,* even though the principal direction of transport was to the electrode of opposite charge. The normalised fluxes of the anionic glutamic and aspartic acids were much greater *in vivo* than *in vitro.* For the zwitterions, two notable differences were observed. First, the magnitude of the normalised fluxes *in vivo* was generally much higher than those seen *in vitro,* implying a much more significant contribution, perhaps, from the human "skin reservoir" to the observed transport. Second, while the preferred direction of electrotransport *in vitro* was clearly towards the cathode, the distinction was much less clear *in* vivo where no obvious bias could be discerned. It should be recalled that, in the present human experiments, to avoid analytical interference problems, there was initially only 30 mM NaCl in the electrode compartments, providing an unbuffered solution at pH ~ 6. The lack of a clear, implied permselectivity in the flux data implies that the net charge on the skin during the course of these experiments was not far from zero, and that the isoelectric point of the membrane was therefore approximately equal to six under the conditions studied.

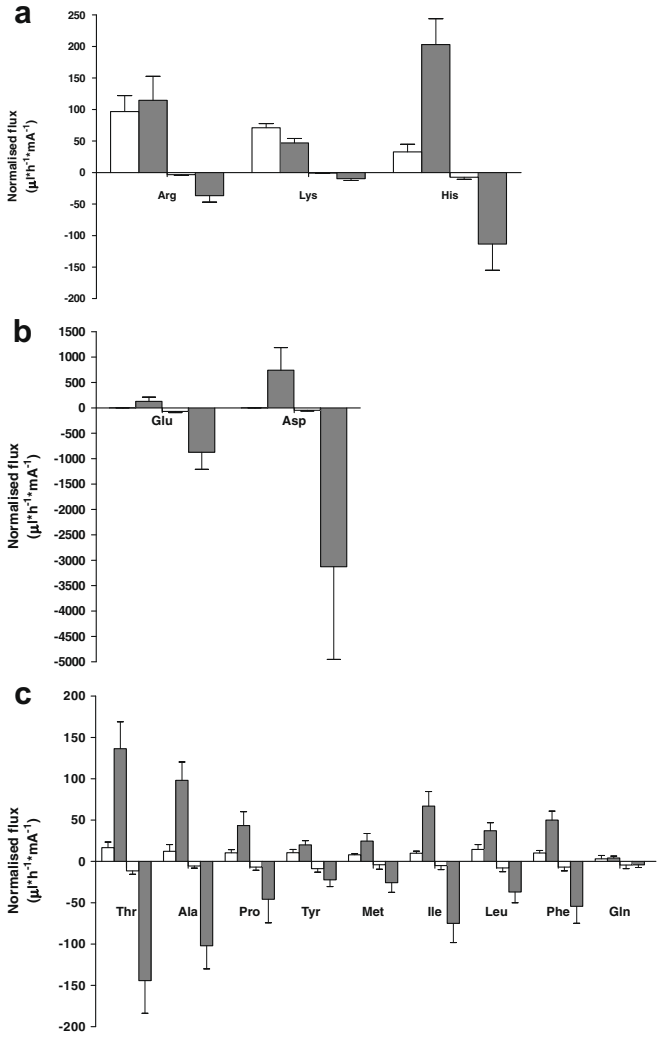


**Fig. 4.** Comparison of *in vivo* (shaded bars) and *in vitro* (open bars [17]) reverse iontophoretic extraction fluxes, normalised by current strength and subdermal concentration. Extraction to the cathode is represented by positive values, and that to the anode is represented by negative values. (a) The data for cationic amino acids, (b) for the anionic species, and (c) for the zwitterions. For clarity, the results for valine, glycine and serine are not included in (c); although the data for these compounds follow a similar trend to the other zwitterions, the normalised fluxes are up to 10 times greater in magnitude.

In summary, this research has shown that it is feasible to use reverse iontophoresis *in vivo* to extract 17 naturally occurring amino acids from and across the skin of normal human volunteers. While the behaviour observed was qualitatively similar to that reported previously [17] in *in vitro* experiments, the levels of amino acids extracted were higher than anticipated, did not reveal a significant permselectivity of the skin, and were not generally correlated with the corresponding plasma levels. It is hypothesised that the existence of a large "skin reservoir" of amino acids (presumably originating from the barrier's NMF [22]) underlies these observations, and further research is in progress to shed more light on this issue.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2008.12.012.

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