Transdermal Extraction of Analytes Using Low-Frequency Ultrasound

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Purpose. Transdermal extraction of clinically relevant analytes offers a potentially non-invasive method of diagnostics. However, development of such a method is limited by the low skin permeability. In this paper, we report a potential method for non-invasive diagnostics based on ultrasonic skin permeabilization and subsequent extraction of interstitial fluid (ISF) across the skin.

Methods. In vivo experiments were performed using Sprague Dawley rats to assess ultrasound-induced skin permeabilization and subsequent extraction of various analytes. Serum and ISF concentrations of various analytes were measured.

Results. Application of low-frequency ultrasound rapidly increased skin permeability. Skin remained in a state of high permeability for at least three hours. During this period, application of vacuum extracted ISF across rat skin in vivo at a rate of 25.7 $\mu l/cm^2/hr$. We measured concentrations of various analytes including glucose, albumin, calcium, urea, triglycerides, lactate, and dextran in transdermally extracted fluid. The composition of the fluid extracted transdermally is similar to that of ISF.

Conclusions. Application of low-frequency ultrasound allows skin permeabilization and extraction of ISF across the skin.

KEY WORDS: ultrasound; sonophoresis; transdermal; diagnostics; interstitial fluid.

INTRODUCTION

The development of rapid, convenient, accurate, and painless methods of measuring blood analyte concentrations has become an area of increasing interest especially for glucose (1). A number of different technologies have been investigated employing invasive, minimally invasive, and non-invasive techniques. Examples of invasive techniques that have been studied include implantable glucose sensors located in blood vessels, subcutaneous tissue, and in the peritoneal (2) cavity. Examples of minimally invasive techniques include the use of lasers to introduce a small hole in the skin through which blood or interstitial fluid can be withdrawn, and lancets that penetrate only into the epidermis and extract a small sample of interstitial fluid (1). Among the non-invasive techniques that have been studied, near-infrared spectroscopy has received much attention as a potential method of glucose sensing (3).

Non-invasive transdermal extraction of analytes offers an attractive alternative to above methods. However, this approach is limited by the low skin permeability to analytes. The enormous barrier properties of human skin are attributed to the stratum corneum (SC), the outermost layer of the skin (4). A variety of approaches have been suggested to enhance transdermal transport of molecules. These include: i) use of chemicals to modify the skin structure, ii) application of electric fields (5,6), and iii) application of ultrasound (sonophoresis) (7,8). Although most studies with these enhancers have been performed with the objective of transdermal drug delivery, iontophoresis (9) has been attempted for transdermal extraction of glucose.

We have recently shown that application of ultrasound can be used for transdermal glucose extraction in human volunteers (10). Specifically, ultrasound was used to enhance skin permeability followed by transdermal extraction of glucose and other analytes using vacuum. In this paper, we assess the generality of this approach for several small as well as large molecular analytes.

MATERIALS AND METHODS

In Vivo Experiments

All animal procedures were performed using institutionally approved protocols. Sprague Dawley rats were used as an animal model since they have been suggested to be a better model than hairless rats for studying transdermal transport of hydrophilic molecules (11). Rats (Sprague Dawley, either sex) were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) injected i.p or i.m. After anesthesia was confirmed, an Ag/AgCl electrode was inserted subcutaneously to measure skin electrical conductivity. A flanged glass cylinder (Crown Glass Co., diameter 15 mm, height 2 cm) was glued on the rat's shaved lateral flank using a minimal amount of cyanoacrylate adhesive (Permabond International or Vet Bond) on the outer edge of the flange. The chamber was filled with 2 ml of PBS and was hydrated for 1 hour. At the end of hydration, the chamber was emptied and filled with 2 ml of fresh PBS or a 1% solution of sodium lauryl sulfate (SLS) in PBS. Ultrasound was then applied using methods described later (Figure 1A). At the end of sonication, the chamber contents were removed and replaced (after rinsing) with 1 ml of fresh buffer (PBS). Extraction was performed through the sonicated site several times using one of the three methods (Fig. 1B): i) passive diffusion, ii) application of low intensity ultrasound (1 W/cm^2, 5 seconds ON 5 seconds OFF, VCX 400, Sonics and Materials), or iii) vacuum (10 in Hg, Air Cadet, Cole Palmer) for 15 minutes in separate experiments. At the end of extraction, the chamber content was collected and analyzed for analyte concentration using methods described in IV. Skin permeability was calculated (assuming steady state) from the equation $P = J/C_S$, where, J is measured transdermal flux, and C_s is the analyte concentration in the serum. Note that this permeability corresponds to diffusive permeability in the case of extraction using diffusion and hydraulic permeability in the case of extraction using convection (vacuum or ultrasound). In this paper, the term "permeability" is used indistinguishably to denote

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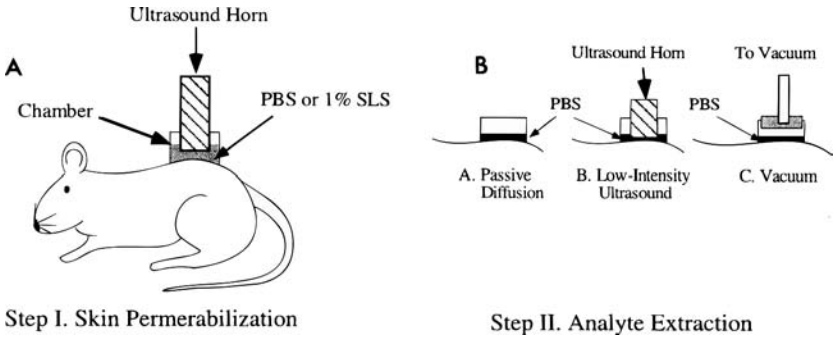

Fig. 1. A schematic representation of ultrasound application and glucose extraction. Figure 1A shows the application of ultrasound while Figure 1B shows the extraction procedure.

diffusive or hydraulic permeability. In some experiments, radiolabeled analytes including glucose, urea, calcium, bicarbonate, dextran, and albumin were injected intravenously through a catheter placed in the jugular vein. These analytes were either ^{3}H or ^{14}C labeled except albumin which was ^{125}I labeled (all obtained from NEN or ARC Research Products).

Ultrasound Pretreatment

Ultrasound pretreatment was performed only once to each skin site in vivo for typically less than five minutes using a sonicator (VCX 400, Sonics and Materials) operating at a frequency of 20 kHz (1 cm from the skin). The sonicators were operated in the pulsed mode (5 seconds on, 5 seconds off). Measurement of ultrasound intensity was performed using a calorimetric method and is described elsewhere (12).

Electrical Resistance Measurements

The skin conductivity was measured between an electrode (E242, Invivo Metric) inserted under the skin and the metal sonicator horn which was immersed in the solution present in the chamber. In order to measure the electrical conductivity of the skin, a 100 mV AC electric field (10 Hz) was applied across the skin for a short time (typically 5 seconds) using a signal generator (model HP 4116 A) and current was measured with an ammeter (Micronta, Tandy Corporation). The electrical resistivity was then calculated from Ohm's law. Skin's specific resistivity was obtained by multiplying the skin electrical resistance (measured experimentally) by the skin area (1.7 cm^2). Skin conductivity was calculated by taking the reciprocal of the resistivity.

Analytical Methods

Concentrations of glucose, urea, triglycerides, lactate, calcium, protein was measured using Sigma Assay kits (catalogue numbers 315, 535, 339-10, 735-10, 587, and 625-2, respectively). The kits are designed to measure physiologically relevant concentrations of analytes in serum. In order to increase the sensitivity of the assay, the Sample:Reagent ratio was modified in each case. The modified ratios were 0.4 for glucose, triglycerides, bicarbonate, lactic acid, protein, and calcium, and 0.08 for urea. Concentrations of dextran, and albumin were measured using a Scintillation Counter (Packard 2000 CA). Serum concentrations were measured by taking a blood sample from cardiac puncture and separating serum by centrifugation. Interstitial fluid (ISF) was collected using the suction-blister method as decribed in Ref. (13). Briefly, a suction cup was designed having an area of 1.7 cm^2 and having four holes each having an area of approximately 0.2 cm^2. Vacuum was applied at 15 in Hg for about 2 hours followed by puncturing the skin with a 30-G needle to collect ISF. Analytes are numbered in the paper as follows: 1. Glucose, 2. Albumin, 3. Calcium, 4. Urea, 5. Triglycerides, 6. Lactate, 7. Dextran.

RESULTS AND DISCUSSION

Skin Permeabilization Using Ultrasound

Ultrasound (especially at low frequencies) can induce cavitation in and around the skin. Oscillation and collapse of cavitation bubbles disorder the lipid bilayers of the skin, which results in enhanced skin permeability. Ultrasound-induced skin permeabilization was monitored using skin conductivity. Conductivity of rat skin prior to ultrasound is about 0.01 $(k\Omega - cm^2)^{-1}$. Fig. 2 shows the change in rat skin conductivity during ultrasound application to the skin when the coupling medium was PBS (open circles). The x-axis corresponds to the total energy, E, delivered to the rat skin ($E = Itd$, where, I is ultrasound intensity (W/cm^2), t is exposure time (seconds), and d is the duty cycle (expressed as a fraction)). The Figure shows that

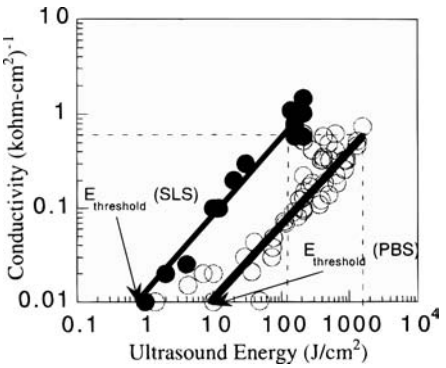

Fig. 2. Effect of ultrasound on the conductivity of rat skin. Open circles correspond to the use of PBS in the coupling medium, while closed circles correspond to the use of SLS in the coupling medium. The horizontal dotted line indicates the stopping point of ultrasound application.

there exists a threshold ultrasound energy, $E_{threshold}$ (PBS), below which no significant change in the electrical conductivity of skin is observed. However, at energies higher than $E_{threshold}$, the electrical conductivity increases with increasing ultrasound energy dose. Application of ultrasound was stopped when the conductivity achieved a value of about 0.6 $(k\Omega - cm^2)^{-1}$ (shown by the horizontal dotted line). Although, the choice of this stopping point was arbitrary, we found that skin with this conductance allows a 100-fold enhancement of transdermal fluxes of various analytes as explained later. Under typical conditions used (7 W/cm^2, 50% duty cycle) the average ON time required to reach this conductance was 2.5 minutes. This time was further reduced by addition of SLS to the coupling medium. Specifically, addition of SLS reduced the threshold ultrasound energy, $E_{threshold}$ (SLS), required to increase skin conductance by about 10-fold (Fig. 2, closed circles). SLS also increased the effect of ultrasound at a given energy on skin conductance by about 10-fold. The average ON time required to achieve a conductance of 0.6 $(k\Omega - cm^2)^{-1}$ in the presence of SLS was 0.25 minutes. Since, ultrasound was applied at a duty cycle of 50%, the total application time was 0.5 minutes. Note that application of SLS alone for 0.5 minutes did not enhance the conductance of rat skin.

Extraction of Glucose Using Passive Diffusion, Ultrasound, and Vacuum

We measured transdermal glucose flux through ultrasound-pretreated skin using three extraction methods, passive diffusion, low intensity ultrasound, or vacuum. While the enhancement of passive diffusion through ultrasound-treated skin is expected due to ultrasound-induced skin alteration, low intensity ultrasound and vacuum may further enhance flux due to convection. The passive skin permeability to glucose prior to ultrasound application was $4 \times 10^{-4} \pm 3 \times 10^{-4}$ cm/hr. However, the skin permeability to glucose (after ultrasound pretreatment from 1% SLS) measured by passive diffusion was $3.4 \times 10^{-3} \pm 2.5 \times 10^{-3}$ cm/hr (an enhancement of about 9-fold). Application of low intensity ultrasound (1 W/cm^2 at a duty cycle of 50%) further enhanced transdermal extraction of glucose. Glucose permeability during low intensity ultrasonic extraction was $2.6 \times 10^{-2} \pm 1.1 \times 10^{-2}$ cm/hr (an enhancement of about 65-fold). Skin glucose permeability measured during vacuum-based extraction following the ultrasound pretreatment was $3.4 \times 10^{-2} \pm 2.5 \times 10^{-2}$ cm/hr (an enhancement of about 100-fold over control permeability before ultrasound application). Enhanced skin permeability (measured by passive diffusion, low intensity ultrasound, or vacuum) was sustained over several hours after ultrasound application. For example, skin permeability measured by passive diffusion immediately after ultrasound pretreatment of rat skin and three hours later was 3.4×10^{-3} cm/hr and 3.0×10^{-3} cm/hr, respectively. Furthermore, no significant recovery in skin conductivity was observed during this three hour period either. Thus, these data suggest that a short pretreatment by ultrasound permeabilizes skin rapidly and the elevated skin permeability is maintained for at least 3 hours in rats. Skin permeability would eventually recover due to reorganization of lipid bilayers (14). We have previously shown that similar results can also be seen in humans. Specifically, the time variation of skin permeabilities at a given site for a given human subject (measured during vacuum application after ultrasound pretreatment) over a period of four hours was within 20%. However, the skin permeability recovered with time and achieved a value comparable to passive permeability after about 20 hours (10).

The data presented so far shows that among the three extraction approaches studied, vacuum-based extraction was most efficient. In view of this, we performed further studies using the ultrasound-vacuum approach. We tested whether application of vacuum on ultrasound pretreated skin extracts analytes other than glucose. Figure 3 shows the measured concentrations of various analytes expressed as percent of their serum concentrations for glucose, calcium, albumin, urea, lactate, triglycerides, and dextran (the last analyte was injected intravenously) after application of vacuum (10 in Hg) across ultrasound-exposed rat skin for 15 minutes. The concentrations of various analytes in the chamber at the end of extraction are shown in Table 1. The Table also shows transdermal flux, serum concentrations, and interstitial fluid (ISF) concentrations of various analytes. The analytes shown in Fig. 3 cover a wide range of molecular properties including hydrophilicity and molecular weight. For example, the analytes cover a molecular weight range of 62 (urea) to 70,000 (dextran). Concentrations of various analytes in the chamber ranged from 0.2% to 3% of their serum values. Note that the volume of fluid (PBS) in the receiving chamber is 1 ml. Hence the extracted analytes get diluted in this volume.

Table 1. Transdermal Flux, Serum Concentration, and ISF Concentration of Various Analytes Considered in this Study

Analyte	Chamber concentration at the end of extraction in mg/dL (dextran is in dpm/ml)	Serum concentration in mg/dL (dextran is in dpm/ml)	Interstitial fluid (ISF) concentration in mg/dL (dextran is in dpm/ml)	Extraction flux (μg/cm^2/hr) except for dextran which is expressed in dpm/cm^2/hr
Glucose	2.1 ± 1.2	183 ± 45	190 ± 50	52 ± 30
Total Protein	35 ± 22	7400 ± 3700	4860 ± 1450	450 ± 230
Calcium	0.08 ± 0.04	7.4 ± 2.6	3.5 ± 1.7	3.1 ± 1.4
Urea	0.26 ± 0.16	33.1 ± 21	26.0 ± 13.8	6.6 ± 4.1
Triglyceride	0.24 ± 0.088	79.1 ± 3.8	24.6 ± 3.8	6 ± 2.1
Lactate	0.5 ± 0.15	15.9 ± 9	38.6 ± 18	8.9 ± 3.8
Dextran	179 ± 46	13166	17906	44700 ± 11520

Figure 3 shows that the concentrations of analytes in the receiving chamber (relative to their serum concentration) vary from analyte to analyte. Since application of vacuum extracts a sample of ISF and not the serum, it is possible that the difference in chamber concentrations of various analytes (relative to their serum concentration) originates from the differences in their ISF concentration relative to the serum concentration. To assess this hypothesis, we measured concentrations of various analytes in serum as well as in the ISF. Table 1 presents the experimentally measured ISF and serum concentrations of analytes used in this study. As can be seen from the Table, the concentration of analytes in the ISF (relative to their serum concentrations) varies from analyte to analyte. For example, while glucose concentration in the ISF is comparable to its serum value, average ISF concentration of triglycerides in rats is only about 30% of its serum value. On the other hand, the ISF concentration of lactate is about two times higher than its serum concentration.

Figure 4 (closed circles) shows a plot of the chamber concentration of various analytes (% of serum concentrations as shown in Fig. 3) against the ratio of ISF/serum concentration from Table 1 (expressed as %). In general, the chamber concentration of various analytes appears to be proportional to their ISF/serum concentration. The line shows the trend defined by the majority of analytes (all analytes except 3 and 6, that is calcium and lactate respectively). The chamber concentrations of analytes 3 and 6 are higher than that expected based on the trend defined by the remaining five analytes. The deviation of analytes 3 and 6 from the expected trend may be attributed to the contribution to the extracted fluxes from sources other than ISF (referred to as erroneous sources). In other words, erroneous sources correspond to sources of analytes that do not correlate with ISF over the extraction period. For example, erroneous calcium may originate from dried urine residue on rat's skin. This amount of calcium may result in higher-than-expected chamber concentrations of calcium and may explain the deviations of analytes 3 and 6 from the expected trend. To assess this hypothesis, we performed experiments where we injected the animals with radiolabeled glucose, lactic acid, calcium chloride, urea and dextran in separate experiments just prior to extraction and measured the amount of radioactivity extracted during vacuum across ultrasound-treated skin. First of all, if any radioactivity is detected in transdermally extracted fluxes, it would suggest that the extracted analytes originate from the blood (or ISF) since that is the only source of radioactivity in the animal. Furthermore, by comparing the amount of radioactive analytes extracted compared to their non-radioactive counterpart, the relative contribution of the erroneous sources can be calculated. Open circles in Figure 4 show the radiolabeled analyte concentration in the chamber after a 15 minute extraction (relative to its serum concentration) plotted against the ratio of ISF/Serum analyte concentration. All the analytes shown by open circles follow the same trend (shown by the line). The direct proportionality between the chamber concentration and the ISF/serum ratio suggests that the extracted analytes indeed correlate with their ISF concentrations if contributions from the erroneous sources are eliminated. In other words, the deviation of analytes 3 and 6 (closed circles) from the expected behavior shown (shown by the solid line) may indeed be attributed to the erroneous sources.

Since the data in Fig. 4 show that the analyte concentrations in the chamber (relative to serum concentration) are proportional to the ISF/serum concentration ratio, it follows that the composition of the contents of the receiving chamber (corrected for erroneous sources in case of analytes 3 and 6 as described in the Fig. 5) should be similar to that of the ISF. This can be seen in Figure 5 which shows the concentrations of various analytes in the receiving chamber after 15 minutes of extraction (relative to glucose, shown on the x-axis) and the concentrations of the same analytes (relative to glucose) in the ISF (shown on y-axis). The relative concentrations of various analytes in the receiving chamber are similar to those in the ISF ($r^2 > 0.99$). This result suggests that application of vacuum after sonication extracts a small sample of ISF into the chamber. However, the actual chamber concentrations of analytes are much smaller than those in the ISF since the extracted ISF

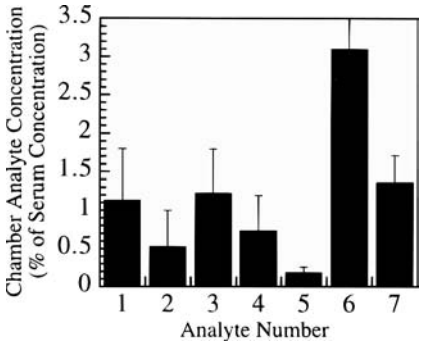

Fig. 3. Chamber concentration of various analytes (relative to their serum concentrations) at the end of 15 minutes vacuum application. The analytes are labeled as: 1. Glucose, 2. Albumin, 3. Calcium, 4. Urea, 5. Triglycerides, 6. Lactate, 7. Dextran. Average ± SD is shown (n = 3–5). Note that the variability in the data is comparable to that observed in the case of passive diffusion.

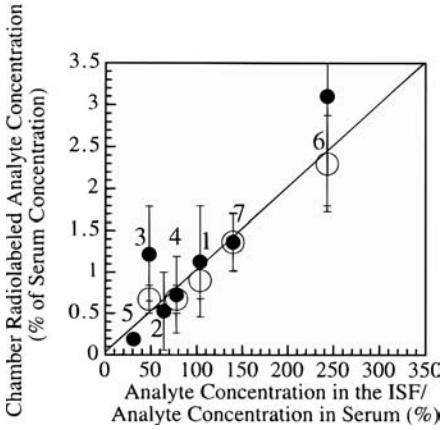

Fig. 4. A plot of chamber concentration of various analytes (relative to their serum concentrations) at the end of 15 minutes vacuum application against the ratio of ISF to serum concentration of various analytes. Errors correspond to SD (n = 3–4). The analytes are numbered as shown in Fig. 3. Open circles show a plot of chamber concentration of various radiolabeled analytes (relative to their serum concentrations) at the end of 15 minutes vacuum application against the ratio of ISF to serum concentration of various radiolabeled analytes. Errors correspond to SD (n = 3–4). The analytes are numbered as shown in Fig. 3.

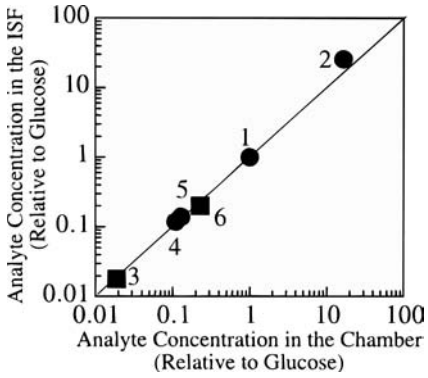

Fig. 5. Relationship between the concentration of analyte in the receiving chamber (relative to glucose) and that in the ISF (relative to glucose). Since the presence of erroneous sources was observed in the case of analytes 3 and 6 (shown by squares) the data for the analytes was corrected using the equation, $C_{\text{corrected}} = \left(\frac{C_{\text{chamber}}^{\text{radiolabeled}}}{C_{\text{serum}}^{\text{radiolabeled}}}\right) C_{\text{serum}}^{\text{unlabeled}}$ where, $C_{\text{chamber}}^{\text{radiolabeled}}$ is the concentration of radiolabeled analyte in the chamber, $C_{\text{serum}}^{\text{radiolabeled}}$ is the concentration of radiolabeled analyte in the serum, and $C_{\text{serum}}^{\text{unlabeled}}$ is the concentration of unlabeled analyte in the serum. Analayte No. 7 is not shown in the Figure since the experiments with this analyte (dextran) were performed only with radiolabeled tracer. Hence the absolute concentration of dextran (relative to glucose) is extremely low.

sample gets diluted in the receiving chamber (volume = 1 ml). The absolute concentrations of analytes observed in the receiving chamber are about 100-fold lower than those in the ISF (Table 1). Since the total volume of the receiving chamber is 1 ml, this implies that application of vacuum extracted about 10 μl of ISF in 15 minutes (the exact volume of ISF calculated from the average chamber concentrations in Table 1 is 10.9 ± 4.2 μl). This corresponds to a convective volumetric flow of 25.7 μl/cm^2/hr. Practical implications of this extracted flux are discussed in Ref. (10).

The data reported here shows that application of ultrasound enhances skin permeability and detectable amounts of interstitial fluid can be extracted through ultrasonically permeabilized skin by application of vacuum for a short period. Concentration of glucose or other analytes in this extracted fluid can be measured using a sensor to potentially develop a continuous monitor of ISF glucose concentration. Further studies should focus on investigating mechanisms of extraction and investigating relationships between glucose concentration in the extracted fluid and blood glucose concentration.

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