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Simple, Miniaturized Blood Plasma Extraction Method

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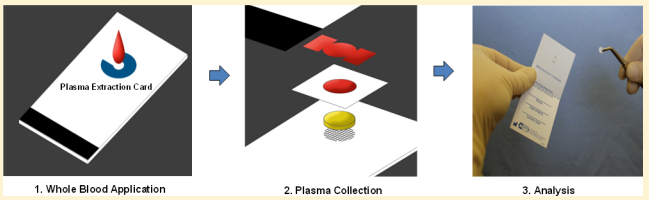
Supporting Information

ABSTRACT: A rapid plasma extraction technology that collects a 2.5 μL aliquot of plasma within three minutes from a finger-stick derived drop of blood was evaluated. The utility of the plasma extraction cards used was that a paper collection disc bearing plasma was produced that could be air-dried in fifteen minutes and placed in a mailing envelop for transport to an analytical laboratory. This circumvents the need for venipuncture and blood collection in specialized vials by a phlebotomist along with centrifugation and refrigerated storage. Plasma extraction was achieved by applying a blood drop to a membrane stack through which plasma was drawn by capillary action. During the course of plasma migration to a collection disc at the bottom of the membrane stack blood cells were removed by a combination of adsorption and filtration. After the collection disc filled with an aliquot of plasma the upper membranes were stripped from the collection card and the collection disc was air-dried. Intercard differences in the volume of plasma collected varied approximately 1% while volume variations of less than 2% were seen with hematocrit levels ranging from 20% to 71%. Dried samples bearing metabolites and proteins were then extracted from the disc and analyzed. 25-Hydroxy vitamin D was quantified by LC-MS/MS analysis following derivatization with a secosteroid signal enhancing tag that imparted a permanent positive charge to the vitamin and reduced the limit of quantification (LOQ) to 1 pg of collected vitamin on the disc; comparable to values observed with liquid−liquid extraction (LLE) of a venipuncture sample. A similar study using conventional proteomics methods and spectral counting for quantification was conducted with yeast enolase added to serum as an internal standard. The LOQ with extracted serum samples for enolase was 1 μM, linear from 1 to 40 μM, the highest concentration examined. In all respects protein quantification with extracted serum samples was comparable to that observed with serum samples obtained by venipuncture.



It is frequently the case that a small, very visible, but unrecognized factor can be a major driver in the emergence of a technology. The future role of mass spectrometry (MS) in clinical diagnostics and personalized medicine is perhaps such a case. The drivers involved, the function MS will play, who will bear the cost, and the degree to which this technology will be available to the developing world are major issues.

Justifiably a great deal of attention has been focused on what MS does, analytical protocols, detection sensitivity, quantification schemes, and regulatory issues. But in so doing we have perhaps failed to recognize a key element; how samples are derived for analysis? Samples come from blood in most cases; drawn in special tubes by a phlebotomist near a laboratory equipped with a centrifuge and blood handling facilities.[1] As the Gates Foundation[2] and others have noted, such amenities are not a worldwide norm. Physicians frequently see their patients in an environment devoid of a blood collection capability. But even when there is, the prospect of the local medical community having the requisite instrumentation for MS analysis of samples is low. Transporting liquid or frozen blood samples to a distant, appropriately equipped analytical laboratory is an alternative, but the expense and difficulty of doing so diminishes the attractiveness of this option; especially transport of frozen samples internationally. The probability an individual at a remote site will enjoy the benefits wrought by an MS analysis of their blood declines in proportion to their distance from a large analytical laboratory.

The focus in this report is on technology that addresses these issues by enabling an individual to extract their own plasma at any location, even in the absence of a power source.

An attractive feature of MS in metabolomics, proteomics, translational medicine, and clinical diagnostics is that microliter sample volumes are sufficient for analysis in many cases. This means that a few μL of plasma from a drop of blood obtained by a finger- or heal-stick would be adequate. Diabetics have taught us that obtaining blood via a finger-stick is trivial and can meet the criteria noted above. The issue is how to extract plasma from a drop of blood obtained in this way.

A series of miniaturized blood fractionation technologies have been reported based on (i) centrifugation strategies,[3,4] (ii)

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filtration and other size discrimination methods,[5−9] (iii) physical removal of cells by a microfabricated comb or weir,[10] (iv) differential diffusion methods,[11,12] (v) dielectrophoresis,[13] (vi) exploitation of the Zweifach−Fung Effect,[14−16] (vii) cross-flow filtration,[17] and (viii) use of acoustic focusing to maneuver cells into small flow channels.[18] Unfortunately none of these technologies meet the criterion of anyone being able to obtain a plasma or serum sample anywhere.

Dried blood spot (DBS) collection on filter paper is another approach, but it lacks blood cell removal and differences in hematocrit negatively impact quantification.[19−24] Hematocrit varies from 20% to 70% based on age,[25] gender,[26] hormonal status,[27] exercise,[28] stress,[29] and diet.[30] As the blood cell to plasma ratio increases, fractionation, and quantification concomitantly becomes more difficult with most of miniaturized methods.[31] The ideal miniaturized fractionation method would be hematocrit independent. Cell type issues are a similar problem. Differences in external properties and deformability of cells can have a strong impact on their removal.[32]

An attractive feature of blood collected on paper is that samples are easy to ship after drying, small volumes can be sampled and transported after drying, and highly compact long-term storage is enabled. The down side of DBS samples from an analytical standpoint is that they are contaminated with variable amounts of components from blood cells that negatively impact analyses by complicating spectra, matrix suppression of ionization increases with contaminants, and sensitivity at low analyte levels is diminished. All of these variables make identification of analytes and their quantification more difficult with blood lysates.

The work reported here focuses on investigating the benefits of collection, drying, and transporting small samples on paper as a dried plasma spot (DPS) after the removal of blood cells and collection of a plasma aliquot. The performance of the plasma extraction card is examined in several ways. The first was to test the variability of volumetric sampling as a function of hematocrit levels. Another was to carry out a vitamin D analysis on plasma samples derived by finger-stick and venipuncture using the same cohort of human subjects. Finally, comparisons were made in protein quantification using venipuncture derived blood spiked with enolase. Results from this comparison study showed plasma from the two sampling methods to be equivalent in both vitamin D and protein analyses.

METHODS

Materials. Iodoacetic acid, proteome grade trypsin, and all other buffer compositions were purchased from Sigma-Aldrich (St. Louis, MO). The C18 spin column was purchased from Nest Group, Inc. (Southborough, MA). 25-Hydroxy-vitamin D_3, 25-hydroxy-vitamin D_2, d_6(26, 26, 26, 27, 27, 27)-25-hydroxy-vitamin D_3, and d_6(26, 26,26, 27, 27,27)-25-hydroxy-vitamin D_2 were purchased from Medical Isotopes, Inc. (Pelham, NH). NoviPlex plasma extraction cards; DR1 (secosteroid signal enhancing tag (SecoSET); 4-substituted-1,2,4-triazolidine-3,5-dione, DR2, and DR3 reagents were obtained from Novilytic (North Webster, IN). Mixed internal standard (IS) was prepared for 25-hydroxy vitamin D2 and 25-hydroxy vitamin D3 analysis, containing 8.2 pg/μL of d_6-25-hydroxy vitamin D2 and 14 pg/μL of d_6-25hydroxy vitamin D3 in methanol. K3-EDTA Vacutainer tubes were purchased from BD (Franklin Lakes, NJ) and used for venipuncture blood collection.

Derivatized Vitamin D Sample Preparation Using the Plasma Separation Device or Liquid−Liquid Extraction. A schematic diagram illustrating the design of the extraction card is shown at Figure 1. About 25 μL of whole blood, obtained using finger-stick, was applied to the "test area" on the top layer of the plasma extraction card. A "control spot" to the side of the application spot changes from white to red as seen in Figure 1, indicating that lateral spreading has occurred and there is sufficient application volume. After three minutes, the top layer of the plasma extraction card is peeled off to expose the *collection disc* which adheres to a *base card* and containing 2.5 μL of the plasma fraction. The sample in the *collection disc* was dried for approximately 15 min, and a 2 μL of mixed IS in methanol was added to the *collection disc* and dried again. The dried *collection disc* was detached from the *base card* and placed into a 2.0 mL eppendorf tube (polypropylene) or stored at −20 °C for later extraction. Extraction was achieved by pipetting 20 μL of methanol directly onto the *collection disc*, then incubated with closure in place for 15 min at room temperature.

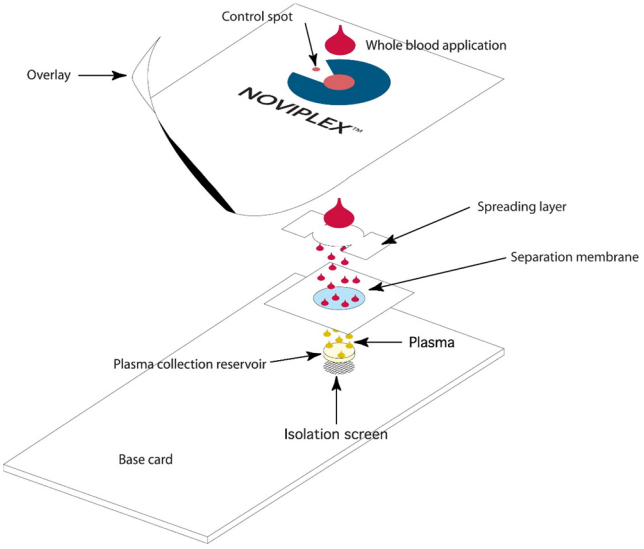

Figure 1. Illustration of the plasma extraction card.

For LLE, venipuncture was used to obtain a specimen of whole blood followed by centrifugation, 10 min at 1000 × *g* to separate plasma from RBCs. 50 μL of mixed IS was added to 100 μL of plasma aliquot along with 50 μL 1.0 M potassium phosphate buffer, pH 7.4. The mixture was combined by vortexing for 20 s then allowed to equilibrate in the dark at room temperature for 90 min. Extraction was performed by adding 1 mL of methyl-tert-butyl ether (MTBE) followed by vigorous mixing for 2 min. The sample was then centrifuged for 10 min at 1500 × *g* for phase separation followed by ∼30 min incubation at −80 °C. The supernatant was collected and transferred to a glass tube, then evaporated to dryness under nitrogen gas at 30 °C.

SecoSET derivatization was accomplished by adding each of three agents (DR1, DR2, and DR3) in sequential order. Twenty microliters of DR1 (4 mg/mL) in methanol (Novilytic L.L.C.) was added to the *Collection Disc* above, followed by vortex mixing for 10 s. Forty microliters of DR2 (2 mg/mL) in methanol was added to the mixture followed by vortex mixing

for 60 s. Immediately after, 40 μL of DR3 (8 mg/mL) in H_2O was added followed by vortex mixing for 10 s. The final solution was transferred to a Qsert vial (Shimadzu, Columbia, MD) and either directly injected into the LC/MS/MS system or stored at −20 °C until analysis. For LLE, SecoSET derivatization was performed in the same manner as described above except: 50 μL of DR1 (2 mg/mL), 50 μL of DR2, and 50 μL of DR3 were used.

Sample Preparation for Proteomic Analyses. Whole blood drawn from a healthy male donor (180 μL) was spiked with yeast enolase (47 KDa) to generate a final concentration of 0, 1 μM, 5 μM, 10 μM, 20 μM, and 40 μM. A 10-fold concentrated enolase stock solution was added at a 1:9 ratio of whole blood. With mild tapping or gentle inversion, the samples were mixed and ∼25 μL of a sample was deposited on the overlay of the plasma extraction card. After saturation, the top layer was peeled off and allowed to dry for 15 min at room temperature. The bottom *Collection Disc* was then transferred to a 2 mL polypropylene tube where proteins were sequentially reduced and alkylated with a modification of methods described in the literature.[33] The remaining blood containing enolase internal standard was centrifuged for 10 min at 1000 × *g* to isolate serum from RBCs. For Proteomics, serum was collected with both the plasma extraction card and centrifugation since venipuncture blood includes anticoagulation agent. A 2.5 μL aliquot of serum was used for further digestion. The samples were reduced with addition of 20 μL of 5 mM TCEP in 50 mM Tris buffer (pH 8.0) for 30 min at 60 °C. Reduced serum samples were alkylated for 30 min at 37 °C in the dark by adding 20 μL of 20 mM iodoacetamide in water. Tris buffer (50 mM) containing 3% ACN, and 1 mM $CaCl_2$ was added to the digests to reduce the reduction and alkylation reagent concentration to 1% (w/v) and to achieve a 200 μL digest volume containing a 1:100 dilution of serum upon addition of trypsin. Trypsin (Sigma) was added to the samples at a 40:1 substrate:enzyme ratio. Digestion was carried out for 18 h at 37 °C. Samples were then acidified by adding a final volume of 0.5% (v/v) formic acid to stop digestion. Samples were desalted and concentrated prior to MS analysis by solid phase extraction using a micro C18 spin column (Nest Group) following the manufacturer's recommended protocol. The eluted samples were vacuum-dried. Prior to LC-MS/MS analysis, samples were reconstituted in 0.1% formic acid, and 3% ACN to a concentration of ∼1 μg/μL based on an initial serum protein concentration of 70 mg/mL.

LC-MRM/MS Condition and Data Analysis for Vitamin D Quantification. Derivatized vitamin samples (∼15 μL) were separated with a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) using a reversed-phase HALO C18 column (150 mm × 2.1 mm, 2.7 μm) at 40 °C and a flow rate of 200 μL/min. The autosampler (SIL-20AC) was maintained at 4 °C. Following equilibration for 5 min with mobile phase A (100% H_2O, 0.05% formic acid) at 200 μL/min, the HALO C18 column was eluted in 6 min linear gradient from 25 to 35% solvent B (100% acetonitrile, 0.05% formic acid) followed by a 8 min linear gradient from 35 to 50% solvent B.

An Applied Biosystems/MDS Sciex 4000 QTRAP with an electrospray ionization source controlled by Analyst 1.4.2 software (ABSCIEX, Framingham, MA) was used in ESI-positive ion mode for all LC-MRM/MS analyses. All acquisition methods used the following parameters: 5,000 V ion spray voltage, a 450 °C interface heater temperature, and Q1 and Q3 set to unit resolution. MRM acquisition methods were constructed using vitamin-specific tuned declustering potential (DP), collision energy (CE) voltages, and retention time constraints. A default collision declustering potential of 85 V, collision exit potential of 6 or 12 V and entrance potential of 10 V was used for all MRM ion pairs. Other MRM parameters are shown in the Supporting Information Table 1.

All MRM data were processed using the Analyst classic algorithm for peak integration. A 5 min retention time window with "report largest peak" enabled was used. The default values for noise percentage and baseline subtraction window were used. All data were manually inspected to ensure correct peak detection and accurate integration.

LC-MS/MS Analysis of Plasma Digests and Database Searching. Peptide samples of 1 μL were introduced with a Zorbax C18 trap column (5 μm, 0.3 mm × 5 mm) in an Agilent 1100 HPLC system coupled to the LTQ Orbitrap XL (Thermo Fisher Scientific Inc.). After switching the trap column in-line, the peptides were transferred to a 75 μm × 150 mm Zorbax 300SB-C18 reversed phase capillary column and eluted with a linear gradient ranging from 5% mobile phase A (0.1% formic acid) to 40% mobile phase B (0.1% formic acid in ACN) over 65 min at a flow rate of 0.3 μL/min. Through a 15 μm ID (75 μm × 360 μm tubing) SilicaTipTM (New Objective (Woburn, MA)), the eluted peptides were electrosprayed into the Orbitrap. While MS1 scans were acquired in the profile mode, a data-dependent acquisition (DDA) was performed to select precursors for MS2 fragmentation with a full MS scan of 300−2000 *m*/*z* at a resolution of 30 000. MS/MS was achieved with scans of the top 8 abundant precursors in a 60 s dynamic exclusion window. Charge status was selected for ≥+2. The normalized collision energy was set at 35.

Xcalibur version 2.0.7.(Thermo Fisher Scientific Inc.) software was used to record the extracted ion chromatograms in a linear ion trap mode and the tandem mass spectra obtained by LC/MS/MS converted to mascot generic format by using Trans Proteomic Pipeline (Institute for System Biology)[34] before exporting to an in-house version of the Mascot server (version 2.3, Matrix Science Inc. (London, UK)) for performing Mascot MS/MS ion searches. Mascot was set up to search the Uniprot/Swissprot databases selected for human or yeast taxonomy. The carbamidomethyl of cysteine and oxidation of methionine were specified as a fixed and variable modification for the search criteria, respectively. Decoy analysis was performed to estimate the false discovery rate (FDR) where protein and peptide FDR were both <0.01. Two missed cleavages were allowed for trypsin digestion. The precursor mass tolerance and the fragment mass tolerance were set to 10 ppm and 0.6 Da, respectively. The searched data through the in-house Mascot server was merged and further analyzed with Scaffold version 3.1.2. (Proteome Software Inc. Portland, OR). Scaffold was used to validate MS/MS based peptide and protein identifications where $p < 0.05$ was considered as a significant peptide confidence, as specified by the Peptide Prophet Algorithm.[35] Protein probability for identifications was assigned by the Protein Prophet algorithm[36] and accepted if the protein probability was greater than 99% with at least two peptides having 95% confidence. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

RESULTS AND DISCUSSION

Plasma Extraction Card Design and Characteristics. The general design of the plasma extraction card used in this

work is illustrated in Figure 1. Not shown in Figure 1 is that the *Spreading Layer* and *Filtration Membrane* components are connected to the *Overlay* using an adhesive while the *Plasma Collection Disc* and *Isolation Screen* are similarly connected through an adhesive to the *Base Card*. Plasma extraction is initiated by applying a drop of blood (25−50 μL) to a *Spreading Layer* through an application hole in the *Overlay*. Lateral spreading is confirmed by the visual appearance of red cells in a small lateral observation window 2 mm away from the sample application site (Figure 1). Based on the time and distance the blood sample travels laterally it can be calculated that the spreading rate averages roughly 150 μm/sec, slowing as the sample reaches the periphery of the spreading layer. It will be seen below that vertical migration into the *Filtration Membrane* in contrast is much slower than lateral spreading. Lateral spreading occurs so quickly that vertical migration through the filtration membrane is essentially as a front. Microscopy shows a decrease in cell density in the spreading layer as the distance from the entry point increases (data not shown). This is interpreted to mean that cells are partially trapped and adsorbed in the spreading layer. The importance of this is that it takes part of the fractionation load off of the semipermeable membrane layer below.

A second level of fractionation below the *Spreading Layer* was achieved in a proprietary porous membrane that has the functions of (i) removing cells from plasma by filtration and (ii) regulating the flux of plasma into the *Collection Disc*. Extraction cards provided by the supplier at early stages of development extracted plasma from blood in less than a min but gave high levels of hemolysis. It was found that reducing the average linear velocity of plasma through the semipermeable membrane and into the *Collection Disc* to 1 μm/sec or less overcame this problem. Sample fractionation time at that velocity is approximately 3 min.

The final layer in the card is a 6.35 mm (1/4 in.) diameter cellulose fiber plasma *Collection Disc* . The rationale for using a disc held up, away from the *Base Card* by an isolation screen is that (i) the disc holds a specific volume of plasma that is independent of the applied blood volume and (ii) wicking of plasma along the *Base Card* surface is precluded. When the disc is saturated with plasma, flow through the membrane system stops; independent of the hematocrit, viscosity, or initial blood volume.

Although flow through the filtration membrane is unlikely to be constant throughout the plasma extraction process, the average loading rate of the *Collection Disc* was 13 nL/sec. This corresponds to a volumetric flow rate into the *Collection Disc* of 400 pL/mm^2/sec. From these values it can be calculated that the average linear velocity of plasma into the *Collection Disc* from the *Filtration Layer* is roughly 1 μm/sec. The very large difference in lateral versus vertical migration is the basis for the conclusion that plasma spreads so quickly it moves through the membrane system in a frontal migration mode.

It is interesting that the linear velocity through the *Spreading Layer* can average 150 μm/sec without causing hemolysis while flow velocity through the semipermeable membrane has to average 1 μm/sec of less to preclude hemolysis. Transport in the *Spreading Layer* is that of cells being carried through a high porosity matrix by plasma. There are not a lot of restricted passages in this membrane. In contrast, cells are retained by size in the *Filtration Layer* and plasma is flowing past them. Red blood cells are known to be shear-sensitive.[37] High flow rates of plasma past stationary cells in the *Filtration Layer* apparently leads to increased hemolysis. Actually increased hemolysis in membranes as a result of high flow rate induced shear has been previously reported[37] and even used as a means to lyse red blood cells.[37]

When the *Collection Disc* had loaded with plasma the top layers of the card were removed, exposing the *Collection Disc* on the *Base Card* to the atmosphere. As noted above, the *Collection Disc* and *Isolation Screen* are attached to the *Base Card* with an adhesive so they do not lift off the *Base Card* during this delamination step. Within 15 min of air exposure the disc appeared to be dry,[38] leaving proteins and metabolites deposited in the cellulose matrix of the disc.

Reproducibility of volume sampling was examined based on data from ten extraction card experiments using blood from the same individual. Immediately following removal of the upper membrane layers the plasma loaded *Collection Disc* was removed from the *Base Card* with a tweezers and weighed on a microbalance. Testing in this study was performed with the 2.5 μL disc (details shown in the Supporting Information). The impact of hematocrit levels of 20%, 41%, and 71% on sample aliquot volume were tested gravimetrically. Intercard variation in aliquots was less than 1% for the 20% and 41% hematocrit samples while that for the 71% sample was 3.4%. (Measured values for these experiments can be found in the Supporting Information Table 2). The %RSD for the three different hematocrit levels was less than 1%. Moreover, with the 41% hematocrit level the %RSD of volume difference between venous blood containing anticoagulation agent (2.52 μL) and finger-stick blood (2.57 μL) was less than 2%.

On the basis of the known concentration of protein in plasma, the 2.5 μL collection volume on a disc could contain up to approximately 200 μg of protein, that is ∼80 μg/μL. Because analytes will later be extracted directly from dried samples on discs, the average thickness of the protein layer on the fibers of the *Collection Disc* is an important question. Paper has long been used in chromatography were polar substances are known to associate with the paper surface by hydrogen bonding. Proteins alone or in a complex can associate with the cellulose matrix as we know from ion exchange and affinity chromatography. Carboxymethylcellulose (CMC) matrices used in ion exchange chromatography are very similar to the cellulose matrix used in the plasma *Collection Disc*. CMC sorbents supplied by Whatman binds 170 μg or more of albumin per μL of sorbent according to the supplier. Protein retention models in liquid chromatography show that protein adsorption at surfaces is essentially in a monolayer.[39] Assuming an equivalent fiber surface area in the *Collection Discs*, protein deposition in the disc would not have to exceed monolayer thickness to accommodate protein deposition. Obviously some proteins will be deposited on cellulose fibers in the disc as aggregates, but only when the aggregate exists in solution before contact with the surface. Adsorption in this case is the result of individual members of the complex dominating the adsorption process. The significance of the protein layer thickness is that organic solvents will be used in subsequent experiments to extract metabolites directly from deposited proteins. The thicker the protein layer, the slower and more difficult it would be to extract metabolites from the disc.

Vitamin D Analysis. Performance of the plasma extraction method was compared with conventional venipuncture sample preparation in the analysis of vitamin D blood levels from the cohort of human subjects. Venipuncture samples were examined by a liquid−liquid extraction (LLE) method using

a commercial vitamin D kit while samples prepared by the plasma extraction method were analyzed by the same method, but adapted to the extraction card. Sample *Collection Discs* bearing dried plasma were removed from the transfer card backing and placed in sample vials where vitamin D was extracted from the dried protein with 20 μL of methanol for 15 min at room temperature and derivatized with SecoSET reagent. SecoSET derivatizes secosteroids through a Diels–Alder addition as seen in Figure 2. The products of SecoSET

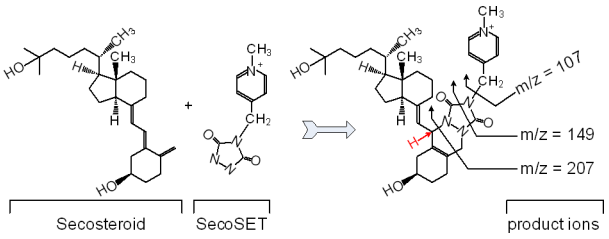

Figure 2. Derivatizaton of a secosteroid with SecoSET. Abundant product ion fragments at m/z 107, 149, and 207 resulting from collision induced dissociation were used in detection.

derivatization of vitamin D isoforms bear a quaternary amine that enhances their ionization efficiency and lowers their limit of detection. Optimum ionization energies and the relative abundance of the major product ions appearing at m/z 107, 149, and 207 varied between the three commercial triple quadrapole instruments (AB SCIEX QTRAP 4000 (used for further study), AB SCIEX QTRAP 5500, and Shimadzu Triple Quadrupole LCMS-8040) used to examine derivatized samples. Quantification was achieved with multiple reaction monitoring (MRM) by adding a known quantity of deuterated internal standard identical in structure to each of the vitamin D isoforms being quantified before derivatization with SecSET and LC-MS/MS analysis. SecoSET derivatized vitamin D isoforms and their internal standards emerged from the reversed phase chromatography column within several seconds depending on the LC conditions of each other during gradient elution. Coelution is important in assuring that matrix suppression of ionization would be identical for each substance being quantified along with its internal standard. While the molecular weight of internal standards was 3 amu higher than the vitamin D isoform being quantified in all cases, the CID product ions derived from fragmentation of the SecoSET reagent were common to all forms of vitamin D and its internal standards. MRM based quantification was achieved by summing the second MS dimension ion current under the elution curve for m/z 107, 149, and 207 of each species being determine. The product ion at m/z 149 was used for quantification since it showed the highest peak intensity while those at m/z 107 and 207 were additionally monitored to make sure the peak was from the desired parent ion. Comparing the total peak areas of the product ions from the internal standard to those from sample vitamin D allowed the concentration of vitamin D and its isoforms to be determined. Peak area counts and linearity in the analysis of vitamin D standard (see Supporting Information for details) are seen in Figure 3 where the limit of quantification (LOQ) was ~400 pg/mL with the 2.5 μL of sample derived from the plasma extraction card. The LOQ with the LLE method was ~10 pg/mL. The 40 fold lower LOQ with the LLE method resulted from the use of a 100 μL sample volume (data not shown).

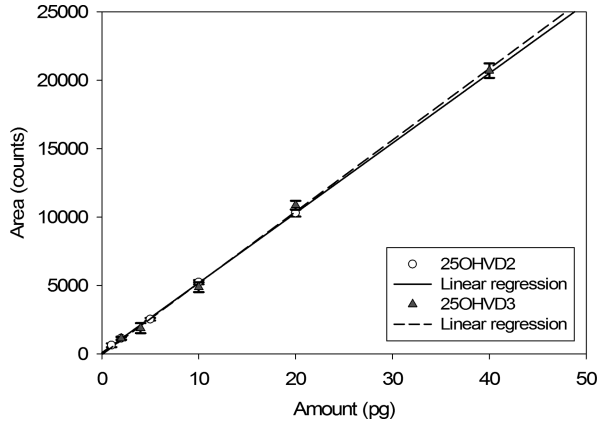

Figure 3. Linearity of response in the quantification of vitamin D isoforms derived from standard vitamins directly deposited on the *Collection Disc* from a plasma extraction card.

Accuracy of the LLE kit using the SecoSET derivatization reagent for quantification was evaluated with a plasma sample (NIST-SRM972a L3) obtained from NIST (Gaithersburg, MD). Vitamins in a 100 μL plasma sample were derivatized with SecoSET and prepared for LC-MS/MS analysis as described in the METHODS. The accuracy of the LLE kit was evaluated relative to values provided by NIST and found to have a % RSD of 0.4% (Table 1) in the total vitamin D concentration between the measurement and NIST value.

Table 1. Accuracy of the Commercial Kit in Determining Vitamin D[a]

NIST-SRM972a L3	LLE100	NIST provided (measured)
25OHVD2 (ng/mL)	13.65 ± 0.41	13.35
25OHVD3 (ng/mL)	19.71 ± 0.64	19.81
total (ng/mL)	33.36 ± 1.05	33.16

[a]The % RSD was 0.4%.

Performance of the plasma extraction method was compared with conventional venipuncture sample preparation in the analysis of vitamin D blood levels from the same cohort of human subjects. A comparison of the time and labor required for vitamin D analysis by the two methods is seen in Supporting Information Table 3. Clearly the plasma extraction method has more desirable features in this respect. Accuracy and reproducibility comparisons were made as well using samples collected simultaneously by both methods from a healthy male. At the completion of plasma collection and drying on the *Collection Disc*, deuterated IS in methanol was added to the disc. After drying again the *Collection Discs* was removed from the *Base Card* with a tweezers and placed in a sample vial. The venipuncture sample was analyzed as described above. A direct comparison of the plasma extraction and venipuncture preparation methods (Table 2) showed that with the same patient cohort % CV values of 4.4 and 1.8 were obtained in triplicate runs, respectively. Vitamin D values seen with the two methods were similar (% RSD < 10%).

Protein Analysis. The rationale in the proteomics studies reported below was to i) examine the linearity of quantification with a standard protein recovered from the plasma collection card while at the same time ii) determine whether any problems were noted at random in the recovery of dried proteins from the paper collection disc. No effort was made to

Table 2. Comparison of the Plasma Extraction Card Based and Novilytic LLE Methods of the Sample Preparation in Vitamin D Analysis

donor 1 (2.57 μL of plasma from finger-stick)[a]	analyte peak area (counts)	IS peak area (counts)	ratio (analyte/IS)	d_6–25OHVD3 (pg on *Collection Disc*)	measured 25OHVD3 (ng/mL)	average concentration of analyte (ng/mL)	% CV of replicates
plasma extraction card, replicate 1	5.07×10^{03}	2.63×10^{03}	1.93	28.0	20.8		
plasma extraction card, replicate 2	5.00×10^{03}	2.74×10^{03}	1.82	28.0	19.6		
plasma extraction card, replicate 3	5.11×10^{03}	2.89×10^{03}	1.77	28.0	19.1	19.8	4.4
donor 1 (100 μL serum from venipuncture)	analyte peak area (counts)	IS peak area (counts)	ratio (analyte/IS)	d_6-25OHVD3 (pg in a tube)	measured 25OHVD3 (ng/mL)	average concentration of analyte (ng/mL)	% CV of replicates
LLE, replicate 1	2.83×10^{05}	8.83×10^{04}	3.20	700	22.2		
LLE, replicate 2	2.79×10^{05}	8.88×10^{04}	3.14	700	21.8		
LLE, replicate 3	3.52×10^{05}	1.08×10^{05}	3.26	700	22.6	22.2	1.8

[a]Plasma volume used for finger-stick was based on Supporting Information.

achieve an in-depth analysis of the plasma proteome. An LTQ Orbitrap mass spectrometer was selected to carry out this work based on the desire to randomly find any proteins in plasma which seemed to give poor recovery. The LTQ Orbitrap is an excellent instrument for this type of study but obviously a triple quadrupole instrument would have given a much lower LOQ with specific proteins. Instruments capable of collecting large numbers of product ions from a small number of specific proteins during the course of analysis will be a better choice in clinical diagnostics where sensitivity is a major issue.

Surprisingly, application of microsampling methods in plasma proteomics has been limited as described in introduction. Among the many sample preparation strategies being used to examine the blood proteome, one of the more popular is the bottom-up approach where proteins in samples are first digested with trypsin and their peptide fragments identified by a combination of reversed phase chromatography and tandem mass spectrometry (MS).[40,41] Amino acid sequences obtained from MS analyses of these peptides are then transcribed to coding DNA sequences and searches of DNA databases used to identify the coding gene and parent protein from which the peptides seen in samples were derived.[42] A refinement of this method is application of a targeted affinity selection method that captures peptides on the basis of their structure.[43,44] The objectives of the studies described here were to evaluate the plasma extraction method in the identification and quantification of a protein in plasma; showing that dried proteins are either solubilized and/or trypsin gains access to the porous membrane matrix inside of the plasma *Collection Disc* whereupon proteins are digested and become available as peptide fragments for LC-MS/MS analysis. This study was conducted by the addition of yeast enolase as an internal standard (IS) protein to a venipuncture derived blood sample. Yeast enolase was chosen because it is not found in plasma and its concentration in a venipuncture sample could be easily controlled.

Venipuncture samples with and without added enolase internal standard were applied directly to the extraction card by pipetting. Enolase containing samples ranging in concentration from 1 to 40 μM were prepared as described in the Methods. Mixing was achieved by gently tapping the bottom of sample vials to minimize hemolysis even though total protein concentration is not impacted by red blood cell lysis.[45] *Collection Discs* bearing dried plasma were transferred to a 2 mL tube and 20 μL of reducing buffer added. Reduction and alkylation of disulfides was achieved as described in the Methods and literature.[33]

Relative concentrations of the enolase internal standard were compared with spectral counting[46,47] to determine the limits of detection and linearity of quantification. Averaged spectral counts are shown in Figure 4 where $y = 2.0x$ of the linearity. Although some deviation in linearity at 5 μM of enolase is seen in Figure 4, overall spectral counts were linear across the

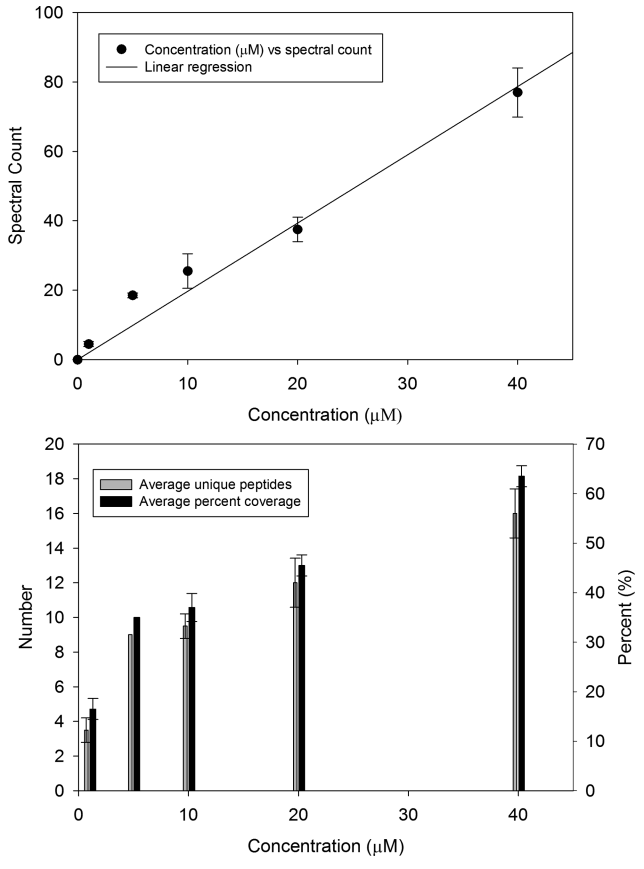

Figure 4. Analysis of enolase internal standard based on peptide level spectral counts, number of unique peptides, and percent coverage with each concentration of enolase which was spiked into whole blood for serum separation and digestion. Top: Spectral count. Bottom: Number of unique peptides and percent coverage.

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concentration range from 1 to 40 μM with a the limit of quantification (LOQ) being 1 μM. It is significant this was achieved without abundant protein removal or any form of target protein enrichment. The number of unique peptides and percent coverage were gradually increased by 4−5 fold from 1 μM to 40 μM. The average variation in enolase concentration was approximately 10% as determined by spectral counting of duplicate samples.

From the ∼1 μg of total serum protein used in the enolase study a total of 59 plasma proteins were observed using identification criteria described in the Methods. Albumin accounts for ∼55% of the total protein in a blood sample and made a good candidate to examine variation in total concentration between runs. From the samples containing enolase at 0−40 μM, the relative standard deviation (% RSD) in albumin was 8.8%. Among the 59 plasma proteins observed 54.8 ± 2.8% of the spectral counts were derived from albumin peptides. Although 59 proteins were identified without depleting abundant proteins, it is highly likely that specific low abundance proteins and metabolites can be determined with antibody based enrichment and high duty cycle instruments such as those using triple quadrupoles, ion traps, or some combination thereof. Sixty two proteins were identified and 60% of the total spectral counts were from albumin with isolated serum obtained by venipuncture while with extracted plasma cards 59 proteins were identified and 55% of the spectral counts were from albumin. Little difference was seen in the data between the two sample preparation methods. Using the plasma extraction card the average number of unique peptides identified and percent coverage with albumin was 45.3 ± 2.7 and 70 ± 2.6%, respectively. With centrifugal isolation of serum the average number of unique albumin peptides was 43.0 ± 2.5, while the average percent coverage was 69 ± 1.8%. In both cases the degree of hemolysis was less than 1% (data not shown). The results of these studies suggest it will be possible with the plasma extraction card to quantify proteins in human plasma with label-free methods and high levels of reproducibility.

CONCLUSION

The argument was made in the introduction that it has been widely assumed that sophisticated analytical tools such as tandem mass spectrometers for the analysis of blood samples must be located close to the point at which blood samples are drawn; the foundation for this idea being that blood samples are difficult to transported to a remote site for analysis. The implications in this hypothesis are that (i) mass spectrometry based clinical diagnostics would only occur in large medical centers, (ii) advanced instrumental methods of analysis would not be available to individuals living far from such centers, or (iii) they would have to travel to such facilities for blood analysis.

On the basis of the data presented here we conclude that the plasma extraction card provides a powerful alternative to venipuncture sampling that has a series of advantages. One is that differential migration of plasma through size discriminating membranes provides a means to remove blood cells from plasma. Moreover this plasma extraction method can be applied by anyone, anywhere, without a laboratory. A second is that the volume of blood required for sampling is far smaller; being of particular significance in neonates, repetitive sampling, and small laboratory animals. A third is that a highly reproducible plasma aliquot is taken during blood sampling. Still another advantage is that dried plasma spots can be transported dry to an analytical laboratory, in a letter size envelope. Yet another is that samples arrive on a small filter paper disc as a protein monolayer ready for analysis. Automated analysis using such sample specimens would represent the ultimate evolution of this work.

Finally we conclude that blood collection technology such as that afford by the plasma extraction card could change the way we think about the future of clinical diagnostics at remote sites and in developing countries. It is being seen in the evolution of modern analytical instrumentation that advances in sophistication are generally accompanied by increases in throughput. This is driving down per sample costs of analysis as we are seeing with large contract research organizations. Putting all this together with the fact that getting the results from most blood tests within a few days is acceptable allows construction of a new analytical paradigm for clinical medicine. It can be suggested that small numbers of very high throughput analytical laboratories to which blood samples are shipped in large numbers by air courier make greater economic sense than trying to place highly sophisticated, very expensive instrumentation at large numbers of sites worldwide. Amazingly the determinant in all this is based on the way we collect blood samples in the future.

ASSOCIATED CONTENT

Supporting Information

Additional materials as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Fred Regnier, Jiri Adamec, and Tim Woenker are founders of Novilytic.

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