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# Distribution of Aβ peptide in whole blood

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**Abstract**

The measurement of amyloid beta peptides (Aβ) in blood and plasma is expected to be a useful biomarker as potential therapeutics designed to lower Aβ peptide enter clinical trials. Many reports have suggested that Aβ could bind to substances in blood that may influence the recovery of Aβ peptide in plasma, its detection by conventional ELISAs or the actual turnover and half-life of the peptide in blood. In this study we describe a process for analyzing total Aβ in whole blood and plasma using denaturing solid-phase extraction followed by reverse-phase HPLC linked to ELISA. Comparison of total Aβ peptide levels in whole blood and plasma from the same bleed showed that most of the Aβ peptide is captured in the plasma if the samples are first denatured. In contrast, plasma that was assayed without denaturation could show greater than 70% reduction in apparent total Aβ peptide. This suggested that there was a pool of Aβ peptide in non-denatured plasma that is occluded from detection by ELISA, perhaps by binding to plasma proteins.


*Keywords:* Aβ peptides; ELISA; HPLC; Blood; Plasma; Denaturation

## 1. Introduction

Pathological levels of amyloid-containing plaques in brain are a principle hallmark of Alzheimer's disease (AD) [1]. The major component of AD plaques are a family of amyloid beta peptides (Aβ), which are derived from the amyloid precursor protein (APP). Considerable genetic, biochemical and neuropathological evidence has implicated either overproduction or impaired clearance of Aβ in the pathogenesis of AD. Many therapeutic strategies designed to lower amyloid production or enhance its clearance are under consideration for further clinical study. Testing the efficacy of these potential drugs in humans will be facilitated by the ability to measure levels of Aβ peptide in routinely available fluids including blood. Currently, this is accomplished by using the plasma fraction of whole blood (e.g. [2–4]). However, potential serious problems to this strategy have been that Aβ forms oligomers that are insufficiently measured by immunoassays alone [5] and a number of plasma proteins have been suggested to tightly bind Aβ and interfere with their detection [6,7]. In addition, platelets in blood may also contribute to the pool of Aβ [8–11] and this material could be lost to the cell pellet when plasma is prepared. The inherently low plasma levels of Aβ, the potential binding by other proteins, and blood cell-associated Aβ are all potential sources of difficulty in simple ELISA measures of plasma Aβ levels.

The current study sought to compare levels of Aβ peptide in whole blood with that in plasma in order to determine if whole blood contained additional pools of this peptide. In order to enable the measurement of Aβ peptides directly from whole blood, denaturing solid-phase extraction followed by HPLC enrichment of Aβ was developed. The system could recover and concentrate Aβ peptides from whole blood or plasma in a quantitative and reproducible fashion and the samples generated by the process were compatible with analysis by ELISA. As a result, the levels of Aβ peptide in plasma could be directly compared to the levels in whole blood or the cell pellet fraction using the same ELISA. The data generated by this process indicated that plasma prepared from normal individuals appears to contain greater than 90% of the total Aβ peptide present in the original whole blood sample. It further demonstrated that plasma Aβ peptide exists in a bound and free state as defined by the ability

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of the ELISA antibodies to detect Aβ in neat plasma compared to denatured plasma.

## 2. Experimental

### 2.1. Materials

Synthetic Aβ 1–38, 1–40 and 1–42 were obtained from Bachem (King of Prussia, PA) and were of 95% purity; [$^{125}$I]Aβ 1–40 was from Amersham Biosciences (Upsala, Sweden). Mouse IgG resin and other fine chemicals, unless otherwise noted, were obtained from Sigma (St. Louis, MO).

### 2.2. Human samples

Blood samples were obtained using healthy individuals that had not fasted from the Jasper Health Clinic, Kalamazoo, MI. Informed consent was obtained from all individuals in the study. Subjects are labeled A–G with the following vital information. A = 45 years old, female; B = 42 years, female; C = 46 years, female; D = 46 years, male; E = 24 years, male; F = 35 years, female and G = 58 years, female.

One hundred milliliters of whole blood was collected in 10 EDTA vacutainer tubes and three of these were transferred immediately into 50 ml polypropylene centrifuge tubes (corning) as 5 ml aliquots and frozen on dry ice. Plasma samples and blood pellets were also prepared from four of the remaining tubes by centrifuging 10 mls of whole blood at ca. 1000 × *g* for 15 min at 4 °C (Beckman–Coulter, Fullerton, CA). The plasma was carefully aspirated from the cell pellet and frozen in either 5 ml or 1 ml aliquots in the same manner as that used for the whole blood. The cell pellets from the 10 ml blood samples were also immediately transferred to the same type of polypropylene tube and frozen on dry ice. All samples were stored at −70 °C and analyzed within 1 month of collection.

### 2.3. Preparation of neat plasma prior to analysis by ELISA

When using non-denatured plasma samples 1 ml aliquots were preabsorbed prior to direct analysis on ELISA in order to lower non-specific background signal. Mouse IgG–agarose resin (1 mg/ml gel, Sigma, St. Louis, MO) was washed twice with 10 volumes of PBS pH 7.5, centrifuged for 15 s at maximum speed in an Eppendorf microcentrifuge and then the pellet was added to plasma that had been thawed on wet ice (0.1 ml resin/ml plasma.). The plasma plus resin slurry was rotated (Scientific Industries, Bohemia, NY) for 18 h at 4° and the resin was removed by centrifugation as before. The decanted supernatant was diluted 1:3 with specimen diluent (10 mM sodium phosphate, pH 7.4, 0.6% human serum albumin, 0.05% Triton X-405 and 0.1% thimerosal) containing 2 M sodium chloride (ICN, Costa Mesa, CA), yielding a final sodium chloride concentration of 0.5 M. This material was used to directly measure Aβ-content in non-denatured plasma using the ELISA (see below).

### 2.4. Extraction of crude peptide pool using solid-phase extraction (SPE)

Five milliliter aliquots of frozen whole blood, plasma or blood cell pellet were vortexed into 46 ml of 6.5 M guanidine hydrochloride, 10 mM sodium phosphate pH 7.2, to thaw and then homogenized using a Polytron (PTA-20S probe) for approximately 30 s (Brinkman Instruments). The denatured samples were diluted to 3 M guanidine HCl by the addition of 49 ml of 0.5% (v/v) phosphoric acid (final pH 2.5). The samples were then re-homogenized for 30 s at low speed and centrifuged at 38,000 × *g* (Beckman–Coulter) for 10 min at 15° in 250 ml bottles in order to eliminate any foam. Only a very small pellet resulted. The supernatant was decanted and re-centrifuged in 100 ml quick seal tubes (Beckman–Coulter) at 48,000 × *g* for 30 min at 15°. Another small pellet resulted. Peptides in the supernatant were concentrated and desalted by solid-phase extraction over two Waters C18 SepPak Plus cartridges coupled in series [12]. The cartridges had been equilibrated prior to use in 0.1% trifluoroacetic acid in water and unbound material was removed by washing cartridges in in the same buffer. Bound peptides were eluted in 0.1% trifluoroacetic acid, 70% acetonitrile in water [12]. Samples were dried in a Thermo-Savant Discovery Speedvac system. The procedure for the recovery of Aβ from solid-phase extraction was optimized using I-125 labeled Aβ 1–40 peptide in parallel experiments.

### 2.5. Reverse-phase analysis of Peptides

Peptide fractions were reconstituted in 500 μl of 25% acetic acid, sonicated in a bath sonicator for 30 min, diluted with 1 ml of 0.1% trifluoroacetic acid, and sonicated for a further 30 min. A small insoluble pellet was discarded after centrifugation in an Eppendorf microcentrifuge at maximum speed, and the supernatant chromatographed by reverse-phase HPLC using an Agilent Technologies 1100 HPLC system, outfitted with a high capacity injection module and integrated fraction collector. Separation of peptide species was carried out on a Vydac 218TP54 column (4.6 mm × 250 mm), at a flow rate of 1 ml/min in 0.1% trifluoroacetic acid, using acetonitrile as the mobile phase. A linear gradient from 25 to 55% buffer B (0.1% trifluoroacetic acid, 80% acetonitrile) over 70 min was used, followed by isocratic elution with 100% B for 10 min. The elution of peptide fractions was monitored by UV absorbance at 214 nm. One milliter fractions were collected and dried overnight in a Speedvac as before. The dried fractions were re-suspended in 500 μl specimen diluent containing 0.5 M sodium chloride each and vortexed for 1 h. Generally, 100 μl aliquots were assayed for total Aβ using the ELISA. Recovery of Aβ peptide from HPLC was determined by spiking samples from the SPE with I-125 labeled Aβ 1–40 and determining the yield of radioactivity in the chromatographic fractions.

### 2.6. Quantification of total Aβ by ELISA

Quantification of Aβ employed a total Aβ sandwich ELISA as described in [13]. Briefly, a high affinity capture antibody

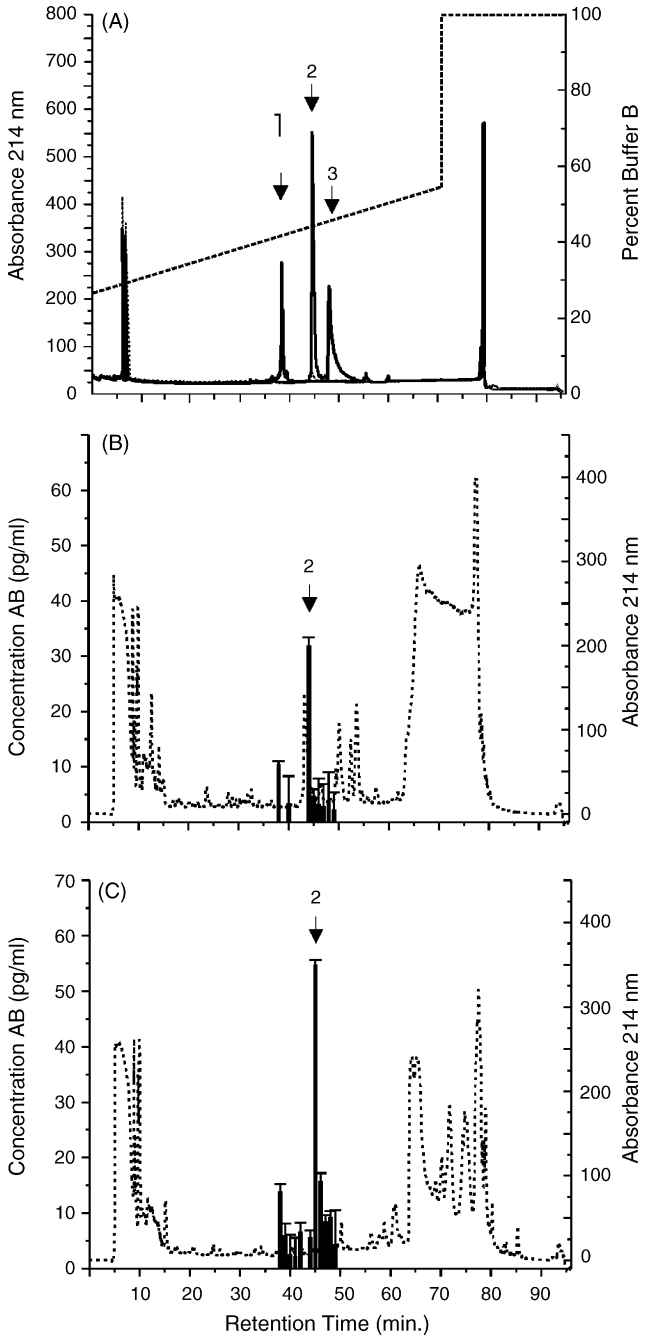


Fig. 1. Panel A: separation of synthetic Aβ 1–37 (peak 1), 1–40 (peak 2) and 1–42 (peak 3) using the system employed for Aβ ELISA linked to HPLC: The resolution of the different Aβ species are sufficient to differentiate them by elution time on RP–HPLC. Under the conditions employed for this study Aβ 1–37 and 1–40 are separated by approximately 6–7 min. while Aβ 1–40 and 1–42 are separated by 2–3 min. Approximately 10 μg of each peptide was injected. Panel B: representative HPLC profile of guanidine hydrochloride-extracted whole blood and the detection of Aβ species by ELISA: 5 ml of frozen whole blood was extracted with guanidine hydrochloride, enriched by solid-phase extraction and developed on reverse-phase HPLC, as described in Experimental. The HPLC profile was monitored at 214 nm. The bars represent Aβ species quantified from HPLC fractions by ELISA using the anti-Aβ (1–5) and anti-Aβ (17–23) antibodies as described in "Methods and Materials". Error bars are standard deviation of two determinations. Panel C: representative HPLC profile of guanidine hydrochloride-extracted plasma and detection of Aβ species by ELISA: 5 ml of frozen plasma was extracted with guanidine hydrochloride, enriched by solid-phase extraction and resolved on reverse-phase HPLC, as described in Methods and Materials. The HPLC profile was monitored at 214 nm. The bars represent Aβ species quantified from HPLC fractions. Error bars are standard deviation of two determinations. The elution position of Aβ 1–40 was confirmed by detecting I-125-labeled peptide and is indicated in panels B and C as peak 2. Note that the Aβ species elute 1 min earlier when in the presence of blood proteins when compared to plasma proteins. This is consistent with the greater protein load associated with blood samples.

(antibody 266 raised against amino acids 13–28 of the Aβ sequence) was coated onto 96 well ELISA plates and the biotinylated Aβ amino-terminal-specific antibody 3D6 was used as the reporter. The biotinylated 3D6 antibody and Avidin–horseradish–peroxidase (Vector Laboratories) were diluted in Specimen Diluent in lieu of casein assay buffer. Antibody binding was monitored with Slow TMB-ELISA HRP substrate (Pierce, Rockford IL) by reacting for 30 min, after which color development was stopped with 1 M $H_2SO_4$. Assay results were quantified in a Molecular Devices Spectramax Plus 384 spectrophotometer by measuring the difference in absorbance at 450 nm and 650 nm. Total Aβ peptide from HPLC-separated samples was determined by summing immunopositive peaks. All such peaks eluted between 30 and 60 min.

## 3. Results

### 3.1. Recovery of Aβ peptide

Each step of the extraction and separation process was analyzed for the recovery of Aβ 1–40 by using synthetic peptide labeled with I-125. Results were from six determinations each of samples from either solid-phase extraction or HPLC. Recovery from SPE samples were 88.15 (±0.85 s.e.) for blood and 94.55 (± 0.36 s.e.) for plasma. Recovery from HPLC was 92.51 (±4.94 s.e.) for blood and 93.91 (±2.14 s.e.) for plasma. This yielded overall recoveries of 81% for blood and 89% for plasma. The recovery was sufficiently reproducible to provide a quantifiable process. In addition to the quantitative properties of the chromatography, it can be observed in Fig. 1A that the HPLC system employed allowed baseline-resolution of Aβ peptides 1–37, 1–40 and 1–42. Therefore, the system was quantitative for total Aβ while at the same time it could differentiate these Aβ species.

### 3.2. HPLC-linked ELISA for measuring Aβ

An example of the combined data from an HPLC-linked ELISA on 5 mls of whole blood or plasma is shown in Fig. 1B and C. Each HPLC fraction can be assayed over a range of dilutions because the sample had been previously concentrated by SPE and HPLC. This allowed for the development of data using the most accurate portions of the ELISA standard curve and it increases the relative sensitivity of the ELISA compared to using tissue or plasma extracts directly. An additional advantage of analyzing samples after enrichment is that interference in the ELISA from blood proteins is minimized due to their removal. It can be noticed that there is more Aβ immunoreactivity per ml in

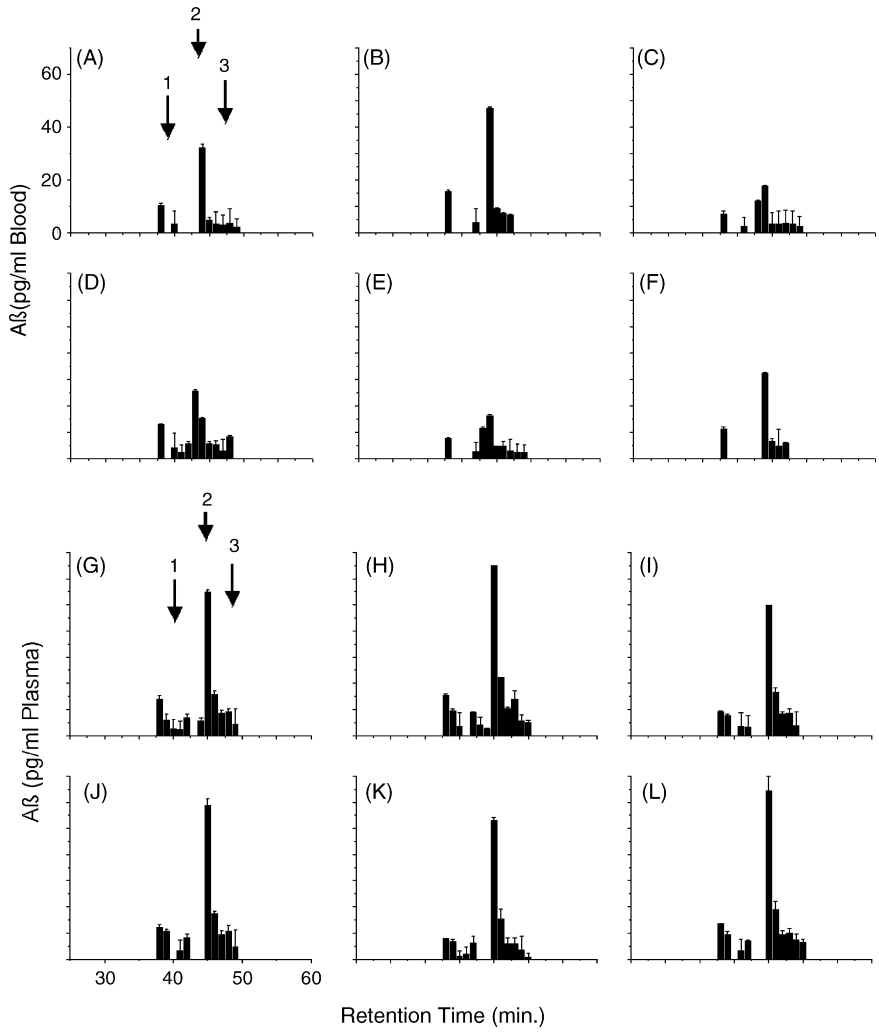


Fig. 2. Panels A through F: recovery of Aβ peptides from guanidine hydrochloride-extracted-whole blood: Aβ species were analyzed from whole blood obtained from six different subjects, using homogenization in guanidine hydrochloride, solid-phase extraction and HPLC as described in Experimental. Aβ species in each HPLC fraction were quantified by ELISA. The relative position of Aβ 1–40 (2) was determined using a radioactively-labeled internal standard in a parallel separation and the position of Aβ 1–37 and 1–42 were estimated based upon the separation shown in Fig. 1. Error bars are standard deviation of two determinations. Panels G through L: recovery of Aβ peptides from guanidine HCl-extracted plasma. Panels G–H correspond, respectively, to the whole blood of subjects in panels A through F. Plasma from each of the six subjects was homogenized in guanidine hydrochloride, captured on solid-phase extraction and separated on HPLC as described in Experimental. Aβ species in each HPLC fraction were quantified by ELISA. The bars represent Aβ species quantified from HPLC fractions. Error bars are standard deviation of two determinations.

plasma than blood from the same bleed and demonstrated how Aβ moves preferentially into the plasma fraction when blood is processed.

The HPLC-linked ELISA data obtained from the whole blood of the six normal subjects can be seen in Fig. 2A through F. The relative position of Aβ peptides 1–37, 1–40 and 1–42 where estimated based upon the retention time of I-125 Aβ 1–40 and the retention times for the synthetic peptides shown in Fig. 1A. The trend from this data showed that Aβ 1–40 is the principle Aβ species in the whole blood from all subjects and that an immunopositive-Aβ specie consistent with Aβ 1–37 is also prominent. Examination of the region where Aβ 1–42 is expected to elute suggested that this Aβ species is not the predominant form in blood which is consistent with previous reports (e.g. [4,14,15]; although see [6]). All samples analyzed in this study came from the same bleeding of each subject so that there is a direct comparison of Aβ peptides in whole blood versus plasma. The process for collecting whole blood or plasma included freezing 5 ml aliquots immediately on dry ice and then thawing the sample into 6.5 M guanidine hydrochloride in order to recover Aβ peptides bound to blood proteins and minimize the possibility of artifactual proteolysis.

The data in Fig. 2G through L demonstrate the profiles of Aβ peptides in the plasma fractions obtained from the whole blood pools in Fig. 2A through F (respectively). The position of Aβ 1–40 was determined using a radioactive tracer and 1–37 and 1–42 were estimated based on relative retention time next to Aβ 1–40. The relative distribution of the Aβ peptide species is similar to that seen in samples of whole blood. Aβ 1–40 is the most predominant form with noticeable signal in a position consistent with Aβ 1–37. Aβ species that elute after 1–40, which includes 1–42, are less abundant. A comparison of total signal in the

Table 1
Comparison of Aβ measured from native or denatured plasma: ELISA for total Aβ was performed either with or without first denaturing the sample in 6 M guanidine HCl

| Subject | Non-denatured plasma (neat) (pg/ml) | Denatured plasma (pg/ml) | Non-denatured:denatured (%) | *T*-test (*p*-value) |
|---|---|---|---|---|
| A | 82.1 (±14.4) | 129.7 (±7) | 63.3 | $(3.0 \times 10^{-7})$[a] |
| B | 154.8 (±9.2) | 165.9 (±3) | 93.3 | $(1.1 \times 10^{-1})$ |
| C | 47.5 (±14.5) | 110.2(±4) | 43.1 | $(6.2 \times 10^{-6})$[a] |
| D | 68.8 (±18.9) | 134.8 (±3) | 51.0 | $(8.9 \times 10^{-5})$[a] |
| E | 37.5 (±9.9) | 107.8 (±5) | 34.8 | $(2.5 \times 10^{-8})$[a] |
| F | 32.7 (±10.7) | 149.5 (±9) | 21.9 | $(4.3 \times 10^{-12})$[a] |

Denatured plasma was also enriched on SPE and HPLC prior to assay. This removed the guanidine HCl as well as many large blood proteins. Error is standard deviation from either 21 determinations (non-denatured plasma) or 2 determinations (denatured plasma). Both types of analysis used plasma from the same bleed.
[a] Denotes a highly significant difference in Aβ peptide levels between non-denatured and denatured plasma. *T*-test was carried out with Origin Statistical Graphics Software using a 2-way independent paradigm.

ELISA data from whole blood (Fig. 2A through F) and plasma (Fig. 2G through L) showed a trend towards the enrichment of Aβ species in the plasma fraction.

### 3.3. Comparison of Aβ peptide recovered under denaturing and non-denaturing conditions

The results in Table 1 show a comparison of the Aβ peptide that can be detected in the same plasma by ELISA either with or without extraction using guanidine HCl and HPLC. Of the six normal subjects studied, only one gave comparable values between native and denaturing protocols (subject B). This sample demonstrated nearly the same amount of Aβ immunoreactivity whether measuring in native plasma or denatured material. The remaining five subjects showed highly significant increases in the amount of Aβ peptide that could be measured from the plasma if it was first extracted by chaotrope. Each subject yielded a very reproducible amount of total Aβ peptide in neat plasma but this value could underestimate the total peptide after denaturing-extraction by greater than 70%. This result suggested that there is a pool of total Aβ peptide in many plasma samples that is inaccessible to measurement by ELISA alone. This may reflect peptide that is bound to proteins or has self-aggregated. The HPLC-linked ELISA process described in this study was able to measure sequestered peptide. Interestingly, Aβ levels in native plasma across subjects ranged over 400% while Aβ levels in denatured plasma ranged only 50%. This suggested that total circulating Aβ peptide levels are more similar across normal subjects than unbound Aβ peptide levels.

### 3.4. Aβ peptide in whole blood appears to reside primarily in the plasma compartment

Table 2 compares the data for total Aβ peptide detection between denatured plasma and denatured whole blood samples taken at the same bleed. The subjects in this study were healthy adults that had been enrolled by the clinic and only included in the study if their hematocrit measured within the range between 40 and 60. Exact hematocrit values were not obtained. Thus, the plasma recovered from 2 ml of whole blood could have varied between 0.8 and 1.2 ml. A hematorcit very close to 50 is commonly observed in healthy humans so this value was used as a point from which to develop a comparison between Aβ peptide in the sample of whole blood and the Aβ peptide that was subsequently recovered when the plasma was extracted. This assumption could lead to an approximately 20% error in the estimation of the actual Aβ peptide concentration in a single plasma sample based upon having assumed that all blood samples yielded $1 \pm 0.2$ ml. This implied that if most of the Aβ peptide was recovered in the plasma fraction that an approximately times two concentration of peptide could be expected when recovering the plasma from whole blood.

Although most of the subjects yielded values that were consistent with the above assumption, subject F demonstrated what appeared to be a departure from the normal expectation. If the hematocrit of this subject had represented the high end of the

Table 2
Total Aβ peptide in blood appears to reside primarily in the plasma fraction

| Subject | Plasma (pg/1 ml) | Whole blood (pg/2 ml) | Plasma:whole blood (%) | *T*-test (*p*-value) |
|---|---|---|---|---|
| A | 129.7 (±7) | 125.3 (±3) | 104.0 | 0.591 |
| B | 165.9 (±3) | 181.2 (±12) | 91.6 | 0.328 |
| C | 110.2 (±4) | 114.2 (±18) | 96.5 | 0.847 |
| D | 134.8 (±3) | 175.5 (±4) | 76.8 | 0.011[a] |
| E | 107.8 (±5) | 112.0 (±17) | 96.2 | 0.842 |
| F | 149.5 (±9) | 120.8 (±9) | 123.8 | 0.149 |

Subjects were healthy adults with hematocrits between 40 and 60%. Therefore, if the plasma contained the majority of the Aβ peptide in blood, one ml of plasma would contain approximately the same amount of Aβ peptide as 2 ml of whole blood. Thus, for purposes of comparison the total Aβ peptide recovered in plasma is shown for 1 ml and the total Aβ peptide recovered in whole blood is shown for 2 ml.
All samples were denatured in 6 M guanidine HCl and desalted on solid-phase extraction (SPE). Concentrated samples from SPE were resolved on reverse-phase HPLC. Plasma samples were prepared from the whole blood analyzed in this study. Error is standard deviation of two determinations.
Of the six subjects that were studied, A, B, C, E and F showed little evidence of total Aβ peptide that was not plasma-associated. Subject D showed a distribution between total Aβ in whole blood and plasma that suggested that up to approximately 20% of the peptide could be non-plasma associated. These results are consistent with the expectation that measuring total Aβ in plasma provides a good approximation of total Aβ peptide in circulation.
[a] Denotes a significant difference consistent with a loss of total Aβ peptide in the plasma fraction. *T*-test was carried out with Origin Statistical Graphics Software using a 2-way independent paradigm.

Table 3
Direct measurement of Aβ in plasma and resultant cell pellets suggests that Aβ is recovered well in the plasma fraction

| Subject | Plasma Aβ concentration (pg/ml) | Blood cell pellet Aβ concentration (pg/ml) |
|---|---|---|
| A | 158.5 ± 9.5 | B.D. |
| C | 137.9 ± 8.5 | B.D. |
| D | 136.5 ± 10.6 | 7.7 ± 1.2 |
| E | 181.6 ± 54.4 | 14.7 ± 1.6 |
| F | 134.7 ± 13.7 | B.D. |

Whole blood was collected from subjects A, C, D, E and F on a second occasion and plasma was prepared as before. After decanting the plasma, the cell pellet was also placed on dry ice. All samples were denatured in 6 M guanidine HCl and desalted on solid-phase extraction (SPE). Concentrated samples from SPE were resolved on reverse-phase HPLC. Aβ was determined on HPLC fractions as described in Experimental. Error is standard deviation from duplicate determinations. B.D., below detection.

range (60%), then instead of 1 ml of plasma from 2 ml whole blood (50%) there would have been about 0.8 ml (40%) of plasma per 2 ml of whole blood and a 20% change in apparent total Aβ peptide (pg/0.8 ml versus pg/1.0 ml). The apparent increase in the concentration of total Aβ peptide in subject F is consistent with this source of error in hematorcrit value and demonstrated an inherent source of error when using clinical samples to obtain absolute quantification. As a result, cell pellets were also probed in order to evaluate the levels of Aβ peptide that may be lost from the plasma fraction.

### *3.5. Aβ peptide recovered from the cell pellet following plasma production represented a small portion of total Aβ peptide in blood*

The data in Table 3 characterized the amount of total Aβ peptide that could be extracted directly from the cell pellet after production of plasma. As can be seen from the data in the table, the cell pellet from five subjects contained no more than 8% of the total detected Aβ peptide whereas the plasma fraction contained 90–100% of the detectable Aβ. This is consistent with the data in Table 2 and strongly suggests that nearly all of the Aβ peptide in blood can be captured in the plasma fraction. Thus, plasma appears to be an excellent sample for Aβ analysis and the less complex nature of the plasma fraction when compared to whole blood offers a sample that is more easily processed.

## 4. Discussion

The process described in this study allowed for the direct analysis of Aβ peptides in whole blood. The use of high concentrations of guanidine hydrochloride in the initial homogenization provided a means to rapidly denature proteases or other enzymes that might destroy endogenous Aβ peptides. It also ensured the release of peptide from blood proteins or multimeric complexes so that they could be detected in the ELISA assay. As a result, the level of Aβ peptide in whole blood could be determined and then compared to the Aβ levels present in the plasma fraction. Using this strategy, it was determined that the plasma fraction prepared from healthy volunteers using EDTA collecting tubes contained greater than 90% of Aβ peptide that was detected in the original whole blood sample from the same bleeding. This finding was further endorsed by demonstrating that the cell pellet from several plasma preparations contained less than 10% of the total Aβ peptide compared to that found in the plasma fraction. These results demonstrated that total Aβ peptide could be estimated from plasma if the sample was denatured prior to assay.

### *4.1. HPLC has aided the analysis of Aβ in a number of studies*

An early example of linking antibody assay of Aβ peptide to HPLC enrichment was carried out by Chen et al. [10]. HPLC has also been combined with gel electrophoresis [16] and mass spectrometry [17]. In an attractive evolution of this strategy, Clarke et al. [18] have developed an on-line analysis that couples automated reverse-phase chromatography to detection and quantification by mass spectrometry. However, it was not possible to use whole blood in these systems due to its complexity. The intent of the present study was to provide a means of exploiting HPLC for detecting Aβ peptides present in whole blood. The principle hurdle was to develop a process that could recover and enrich the peptides sufficiently so that the resultant sample could be fractionated on HPLC and measured using immunoassay. This was accomplished by using the well-characterized properties of guanidine HCl as a chaotrope and solid-phase extraction as a capture step (e.g. [19,20]). Guanidine HCl was chosen over organic solvents for extracting Aβ because it offered an improved recovery of radiolabeled Aβ 1–40 (I-125). Organic solvents and dilute acids permitted much of the labeled Aβ peptide to adhere to the protein pellet and be lost (data not shown).

The presence of Aβ peptides primarily in the plasma fraction is in contrast to the observation described in [10]. In that study, platelets contained approximately 90% of the Aβ peptide immunoreactivity whereas plasma contained less than 10%. The reason for this discrepancy is unknown but several operational differences did exist between the present study and the previous one [10]. First, ethanol was used as extracting agent from plasma. In this study ethanol precipitation of plasma caused loss of Aβ peptide to the protein pellet. Also, the assay used direct ELISA on plasma proteins that had been absorbed onto immunoassay plates. The current study used soluble plasma peptides in a sandwich ELISA. This may have improved the detection of Aβ peptides from plasma by not requiring that they be bound in order to be measured. It is also possible that differences in the collection of plasma caused Aβ peptides to be released into the plasma fraction from blood-borne cells. Platelets are a well-characterized source of Aβ peptides (e.g. [11,21]) and stimulation with agonists or perhaps shearing of cells during collection might cause Aβ peptide release into plasma. However, [22] Olsson et al. investigated the effect of platelet stimulation on Aβ peptide levels in plasma and determined that this did not have a major affect on Aβ levels. Based on the current data, if platelets are the primary source of blood-borne Aβ peptides then this is a dynamic process whereby Aβ peptide is rapidly released from platelets soon after it is produced.

### 4.2. Aβ peptide may exist as a complex in blood.

The comparison of Aβ peptide recovered from either native or denatured plasma from the same blood sample suggested that a fraction of the Aβ peptides naturally exist in a bound form that is not accessible for antibody binding. The amount of bound peptide is consistent within a subject but can vary from nearly 0 to 70% across subjects. A number of plasma proteins that complex with Aβ peptide have been characterized. These include α2-macroglobulin and serum albumin [6,23], apolipoproteins J and E [24,25] gelsolin and ganglioside GM1 [26–28] and transthyretin [29]. It has been suggested that the most abundant complex in plasma is with albumin [23], although albumin is used as carrier in the ELISA and it does not appear to interfere with detection. However, Aβ peptide-albumin complexes formed *in vitro* may not be equivalent to the same complex formed *in vivo*. The possibility that the bound Aβ peptide is complexed with either transthyretin [29] or gelsolin or ganglioside GM1 [28] is intriguing. It has been proposed that these complexes impede the movement of Aβ peptides into the CNS from the blood. Therefore, the fraction of bound Aβ peptide in plasma may parallel the risk of developing AD in some cases. Indeed, a recent eloquent study [30] has highlighted RAGE-mediated transport of circulating Aβ peptide as a important factor in the pathogenesis of cerebrovascular β-amyloidosis and the extent to which Aβ peptide is complexed by other proteins could influence the rate at which peptide is moved into the CNS by this mechanism. The ability to study Aβ peptide from plasma in both native and denatured states and an improved understanding of the forms in which Aβ peptide exists in blood will aid our understanding of how this mechanism contributes to the disease.

### 4.3. Limitations of HPLC-linked ELISA for measuring Aβ peptides

The current process was developed for the purpose of directly evaluating if the total Aβ peptide present in plasma was a good estimate of the total Aβ peptide in the circulation. This is an important question since circulating Aβ levels are a clinical pharmacodynamic biomarker for compounds that inhibit Aβ peptide production *in vivo*. To this end the process worked well in that it gave consistent peptide yields and excellent recovery of Aβ 1–40 which is the major Aβ peptide present in blood. However, it is important to note that the current scheme is not characterized for identifying Aβ 1–42. Aβ 1–40 is abundant in blood and eluted just before Aβ 1–42 on reverse-phase HPLC. This caused Aβ 1–42 to elute as a trailing peak on 1–40 in the current system which compromised the quantification of Aβ 1–42. Additionally, elution time alone is not sufficient to identify specific Aβ peptides since similar or modified species could elute similarly upon separation on HPLC. The quantification of Aβ 1–42 on reverse-phase HPLC has been further complicated by the variable quality of commercially available Aβ 1–42 standard peptides. Commonly available standards demonstrate significant levels of byproducts which can complicate measures of peptide recovery on HPLC or interfere in antibody assays. The former situation is compounded by the widespread use of HPLC to purify Aβ 1–42 peptide standards for use as HPLC standards. Consequently, we were unable to rigorously establish the level of recovery of Aβ 1–42 in the current study.

In the current study, the HPLC separation provided a means to concentrate Aβ peptides from whole blood while keeping the protein content of the sample low enough to still function well in the ELISA. This was important for the detection of Aβ peptides in blood and plasma because of their inherently low concentrations in that fluid. The profile obtained by performing the ELISA detection across the fractions collected from HPLC in this system provided a relative picture of Aβ species in the sample.

## 5. Conclusion

The current strategy of linking the detection of Aβ peptides by ELISA to the concentration and separation of Aβ peptides using SPE–HPLC offered some important advantages. First, samples could be concentrated up to 50 times prior to ELISA when 5 ml of blood or plasma was employed. This allowed determining Aβ levels over the entire dynamic range of the ELISA binding curve and it facilitated the detection of minor species. Additionally, the ability to quantify Aβ peptides at specific positions in the HPLC elution profile allows for the comparison of different Aβ peptide species using the same antibody assay. This circumvents the difficulty of using different antibody assays for the purpose of comparing different Aβ species. The process described in this study is not limited to the measurement of Aβ peptides. SPE–HPLC linked to ELISA is amenable for use with other antigens and it offers a powerful method for identifying both abundant and non-abundant peptides. The process is also amenable to automation and it offers the prospect of measuring an entire spectrum of related peptides from a single sample. As a result, SPE–HPLC linked to ELISA can be expected to be an important enabling tool for the discovery of biomarkers in many therapeutic areas.

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