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Competitive ELISA Studies of Neural Thread Protein in Urine in Alzheimer's Disease

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A specific and reliable competitive affinity assay kit has been developed to quantitatively measure neural thread protein (NTP) in first morning urine samples. This assay, called the urine neural thread protein test (UNTP), is a competitive enzyme-linked immunosorbent assay (ELISA) format affinity assay using 32-well microtiter plates. The assay detects UNTP in the 10–60 μg/mL range (an improvement over earlier assays of $10^3\times$), is linear and more reproducible (average coefficient of variation [CV] 6.2% in precision studies). The utility of the assay has been demonstrated in urine samples from patients with Alzheimer's disease (AD) and controls (sensitivity of 90% and specificity of 91%). Test–retest assays of subjects with AD and controls were comparatively stable at intervals of 2 days to 4.5 years, which suggests that positive (elevated) or negative (normal) NTP levels do not fluctuate significantly over time with respect to the cutoff. J. Clin. Lab. Anal. 21:24–33, 2007. © 2007 Wiley-Liss, Inc.

Key words: brain; neuron; neurodegeneration; protein; dementia; diagnosis

INTRODUCTION

Alzheimer's Disease (AD) is the most frequent cause of dementia, afflicting approximately 15 million people worldwide (1,2). The disease is characterized pathologically by prominent atrophy of corticolimbic structures with neuronal loss, neurofibrillary tangle (NFT) formation, aberrant proliferation of neurites, senile plaques, and amyloid deposition in the brain. More than 90% of AD is sporadic. There is an average delay of nearly 3 years from when initial symptoms appear to when AD is diagnosed (3). Apolipoprotein E ε4 allele is a genetic risk factor that is not found in 50% of cases of AD (4). Tau and β-amyloid protein measurements in cerebrospinal fluid (CSF) and serum Aβ have significant overlap between AD and non-AD levels (<60% sensitivity, <80% specificity), limiting their usefulness (5–14). Other potential AD biomarkers have also recently been described, such as 8-hydroxy-2-deoxyguanosine (15) and hemeoxygenase-1 (16), which have yet to be clinically validated. The clinical presentation of AD can vary enormously, and early diagnosis is a significant practical problem for the primary care physician. It is widely recognized that a reliable peripheral biochemical marker is needed to help in the accurate and early diagnosis of AD and for the assessment of therapeutics (17–23).

Neural thread proteins (NTPs) are a group of brain proteins with cross-immunoreactivity, some of which are modulated during development, and others are related to injury (24–26). The AD related neural thread protein (also referred to as AD7C-NTP, or "NTP" for short) has a predicted 375–amino acid sequence that was derived from cDNA extracted from postmortem AD brain (24). NTP is believed to be a membrane-spanning Alu-containing phosphoprotein, although its exact structure and function are not yet fully understood. NTP is overexpressed in AD beginning early in the course of the disease, and NTP protein accumulation in neurons colocalizes with phospho-tau immunoreactivity (25,26). Induction of NTP gene expression results in increased cell death mediated by apoptosis, impaired mitochondrial function, and increased cellular levels of p53 and CD95 proapoptosis gene products, as occurs in AD (27). Overexpression of NTP is associated with increased levels of phospho-tau. NTP overexpression

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probably has a direct role in mediating some of the important cell-death cascades associated with AD neurodegeneration (25–27). An earlier sandwich laboratory assay (not a kit) for NTP using monoclonal capture and polyclonal detection antibodies was used in studies of AD urine and cerebrospinal fluid (24), but had certain drawbacks, including lower signal detection (maximum NTP level <100 ng) and manufacturing variability of recombinant protein standard material. We have developed a new and reliable competitive enzyme-linked immunosorbent assay (ELISA) kit assay for NTP to help in the diagnosis of AD which overcomes the above problems, and which detects NTP in AD urine in the 10–60 μg range. This report presents the assay and its analytical characterization.

MATERIALS AND METHODS

Materials

The kit consists of:

- Microcon YM-10 Centrifugal Filter Device, (Millipore, Billerica, MA) (one bag containing 11 filters).
- Microcon YM-100 Centrifugal Filter Device, (one bag containing 11 filters).
- Coated microtiter plate (one foil pouch containing four strips in holder). (Refrigerate 2–8°C)
- Trisma buffered saline pH 7.0 solution, 25 mL (Sigma-Aldrich, St. Louis, MO), Nalgene bottle (Nalgene, Rochester, NY). (Refrigerate 2–8°C)
- Wash Buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl [TBS] with 0.05% Tween-20), 25 mL, Nalgene bottle. (Refrigerate 2–8°C)
- Processing Buffer (10 × TBS with 0.5% sodium azide), 50 mL, Nalgene bottle. (Refrigerate 2–8°C)
- Paranitrophenol phosphate (pNPP) solution, 6 mL, Nalgene bottle. (Refrigerate 2–8°C)
- Bovine serum albumin (BSA) powder, 20 mg, borosilicate vial. (Refrigerate 2–8°C)
- Standard Diluent (deonized water; Poland Spring, Wilkes Barre, PA), 10 mL, Nalgene bottle. (Refrigerate 2–8°C)
- Ammonium hydroxide 5% solution, 100 μL, boro silicate vial. (Room temperature)
- NTP Standard powder, 2 mg, borosilicate vial. (Refrigerate 2–8°C)
- Alkaline phosphatase (AP) Conjugate Diluent (TBS w/0.05% Tween 20), 20 mL, Nalgene bottle. (Refrigerate 2–8°C)
- AP conjugate in TBS 20 μL, borosilicate vial. (Refrigerate 2–8°C)
- High NTP human urine control (frozen), 600 μL, borosilicate vial. (Keep frozen $\leq$–20°C)
- Medium NTP human urine control (frozen), 600 μL, borosilicate vial. (Keep frozen $\leq$–20°C)
- Low NTP human urine control (frozen), 600 μL, borosilicate vial. (Keep frozen ≤ -20°C)
- Microcon Microcentrifuge tube for use with Microcon Filters (one bag containing 22 tubes).
- Balancing unit (Microcon Microcentrifuge tube with Microcon Filter attached) (one bag containing one tube and filter).

Equipment

- Centrifuges: Fisher Scientific Marathon 16K/M Microcentrifuge (Fisher, Pittsburg, PA)
- ELISA reader: Bio-Rad Model 550 Microplate Reader (Bio-Rad, Hercules, CA)
- Plate Washer: Nunc-Immunowash 12-wash block
- Micropipettors and multichannel pipette

Sample Collection and Preparation

From a first morning void, 30–50 mL of urine is collected in a clean urine collection cup. A non–first morning sample invalidates the assay. Urine can be processed immediately or refrigerated at 2–8°C up to 48 hr before processing, provided it is not contaminated or otherwise excluded. If the urine sample is to be transported it should be kept refrigerated (2–8°C) at all times, and a Stabilur tablet (Cargille Laboratories, Cedar Grove, NJ) should be added to the sample. The NTP test is not valid on frozen urine.

Specimen Processing

1. A routine urinalysis and creatinine measurement is performed using Roche Chemstrip (Roche Diagnostics, Indianapolis, IN) or equivalent method. If the urinalysis has any abnormality or if the creatinine is <50 mg/dL or >225 mg/dL, a new first morning urine sample should be obtained from the subject.
2. A total of 30–45 mL of urine is centrifuged in a 50 mL tube at 3,000 *g* for 15 min to remove cellular debris.
3. The urine is then filtered through a 0.22 μM cellulose acetate filter using a 50 mL collection tube.
4. 1:10 volume Processing Buffer (0.5% sodium azide in 10 × TBS) is added to the filtrate in order to bring the filtrate to 0.05% Azide in TBS ("F+P" = filtered and preserved).
5. 0.5 mL of F+P labeled urine and the urine controls are placed into the top of Microcon YM-10 filter unit, and centrifuged at 10,000 RPM (12,100 g) for 30 min.
6. The Microcon YM-10 is removed from the centrifuge. The pass through (liquid in bottom of unit) is discarded.

7. The retentate (material above filter) is reconstituted to approximately 0.5 mL with 1 × TBS and centrifuged at 10,000 RPM (12,100 g) for 15 min. The pass-through is discarded.
8. The retentate is reconstituted to 0.5 mL with 1 × TBS, and centrifuged at 10,000 RPM (12,100 g) for 15 min. The pass-through is discarded.
9. On a balance a YM-100 filter unit is weighed, and the retentate is added to the top of the unit and sufficient TBS is added to bring the mass to 0.5 ± 0.01 g.
10. The YM-100 is centrifuged at 10,000 RPM (12,100 g) for 5 min. The pass-through can be tested immediately or stored frozen at −20°C for up to 1 year before testing. Processed samples cannot be freeze-thawed more than twice, and samples frozen prior to processing cannot be used.

The Assay

The assay is performed according to a detailed Clinical Laboratory Improvement Amendments (CLIA)-approved Standard Operating Procedure.

Principle of the Procedure

The assay is a competitive affinity assay that detects NTP. NTP in patient urine sample, control or standard competes with an AP-labeled rabbit immunoglobulin G (IgG) for binding. In the absence of NTP, the plate binds the AP conjugate and absorbance is high. In the presence of NTP, binding is decreased in proportion to the amount of NTP present. The sample concentration of NTP is read off the curve generated by the standards.

Quality Control

Controls should be processed like a sample and run on each plate. Urine controls cover three ranges of analyte, namely a high control, a medium control, and a low control. Two of the three controls must be acceptable for the assay run to be acceptable.

Protocol

1. Add 60 μL of ammonium hydroxide to 10 mL Standard Diluent.
2. Add 2 mL of Standard Diluent with ammonium hydroxide to standard powder. Mix well (by inverting 10 times) and let stand for at least 1 hr.
    a. Dilute Standard to 80 μg/mL by adding 0.2 mL to 2.3 mL of 1 × TBS and mix well.
    b. Dilute 1 mL 80 μg/mL Standard with 1 mL of 1 × TBS to make 40 μg/mL Standard and mix well.
    c. Repeat dilution with 1 mL of 40 μg/mL to 1 mL TBS to make 20 μg/mL and repeat dilution with 20 μg/mL to make 10 μg/mL Standards. Mix standards well after each step.
    d. Use TBS only for 0 μg/mL Standard.
3. Thaw Controls and bring to room temperature for 30 min.
4. Remove the microtiter plate and all buffers from the refrigerator.
5. Add 20 mg BSA to the bottle containing 20 mL AP Conjugate Diluent and invert to dissolve.
6. Add to the AP Conjugate vial 200 μL (Step 5) of the AP Conjugate Diluent with BSA, cap vial, and invert 10 times. Add back the 200 μL to the 19.8 mL of AP Conjugate Diluent (Step 5) with BSA, cap, and invert to mix.
7. Add 50 μL of Standard, Control, or sample to the wells using a single-channel pipette; then add 50 μL of AP Conjugate to wells of the plate using a multichannel pipette.
8. Incubate 1 hr at room temperature, covered.
9. Dump plate. Bang on paper towels and wash with Wash Buffer using a multichannel pipette (200 μL per well). Repeat 2 × for a total of three washes.
10. Add 150 μL of pNPP and allow the assay plate to develop.
11. Check the optical density (OD) at 405 nm after 10 min and, if necessary, thereafter every 3–5 min until the OD of the 0 μg/mL (TBS) Standard is between 1.9 and 2.1.
12. Read concentrations results off the semilog curve.

Results

1. Results of the patient sample, urine controls, and the standards on the plate reader are read using a semilog curve. Test results are calculated in concentration of NTP in μg/mL from a standard curve generated by a semilog fit to the absorbance values of the respective standards.
2. Acceptance criteria: both 0 μg/mL (TBS) Standards must develop to at least 1.9 OD at 405 nm within 120 min after the addition of pNPP to the plate.
3. For each of the high, medium, and low-NTP Controls, two NTP μg/mL values are determined and the mean of the two results is calculated.

Acceptance criteria (Fig. 1): Acceptable if two out of the three NTP Control mean values are within range, that is:

1. High-NTP Control mean value is between 47–71 μg/mL; and/or

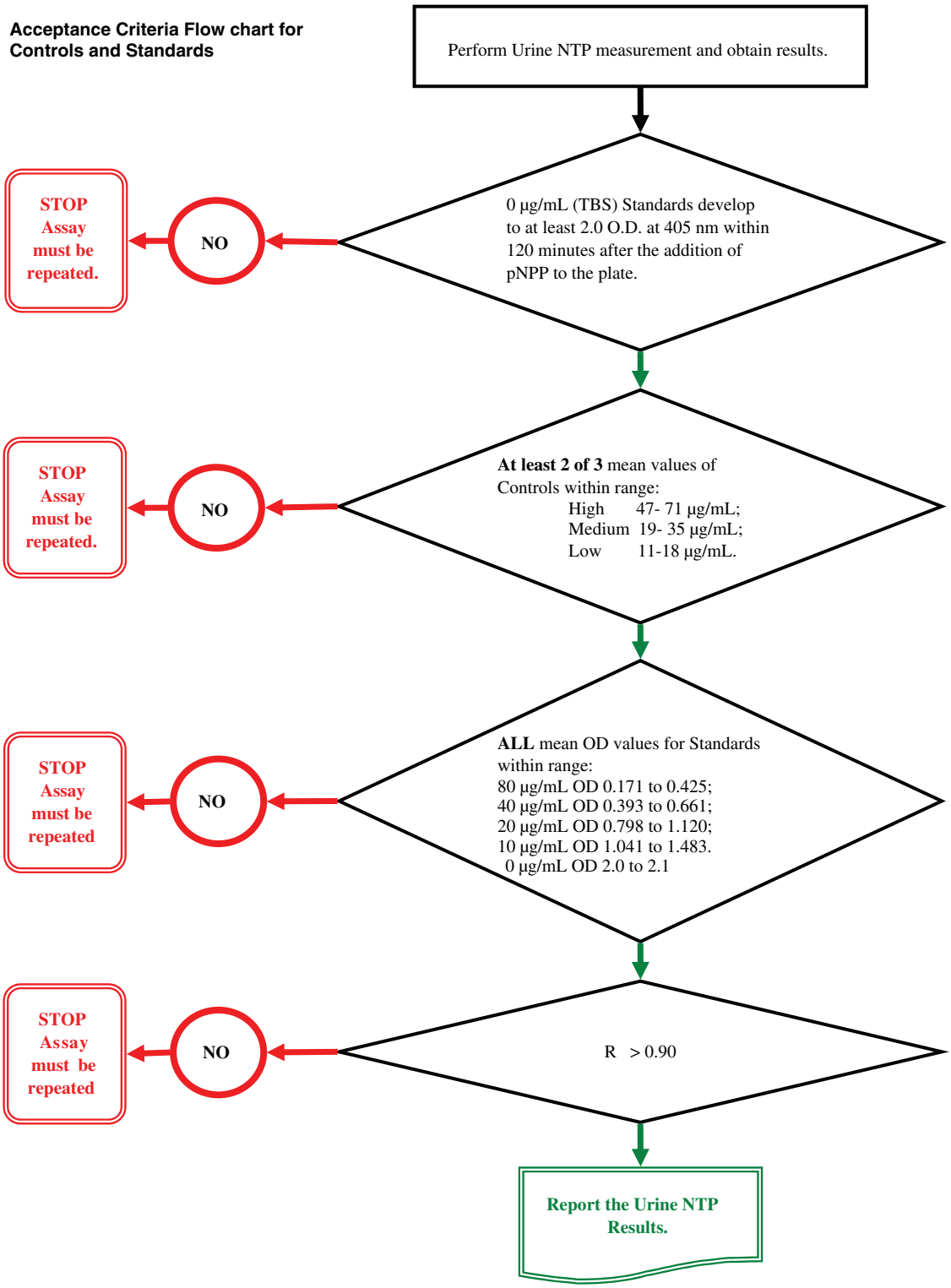

Fig. 1. Flowchart of acceptance criteria for assay.

2. Medium-NTP Control mean value is between 19–35 µg/mL; and/or
3. Low-NTP Control mean value is between 11–18 µg/mL.
4. For each of the Standards (80, 40, 20, 10, and 0 µg/mL (TBS) Standards), two OD values (405 nm) are determined and the mean value of the two results are calculated. Acceptance criteria: Acceptable if:

- 80 μg/mL Standard mean OD value is between 0.171–0.425;
- 40 μg/mL Standard mean OD value is between 0.393–0.661; AND
- 20 μg/mL Standard mean OD value is between 0.798–1.120; AND
- 10 μg/mL Standard mean OD value is between 1.041–1.483; AND
- 0 μg/mL (TBS) Standard mean OD value is between 1.9–2.1.

5. R value and R^2 value are calculated for the curve fit for the semilog curve. Acceptance criteria: Acceptable if:
   a. $R \geq 0.9$; AND
   b. $R^2 \geq 0.81$
6. The patient NTP results are reported if all Acceptance Criteria are met. Otherwise, the assay is repeated.

The microplate reader's data analysis program calculates the coefficients of variation (CVs) for standards, controls, and samples, plots the standard curve, constructs a linear curve fit equation, and calculates the NTP concentration for all samples.

Clinical Study

A total of 14 individuals were tested with repeated samples at intervals of 2 days to 4.5 years

1. AD: Seven individuals with AD provided samples after IRB approval (courtesy of Dr. G. Golden, Coatesville, PA, VA Center, and Thomas Jefferson University Medical School). Each case was characterized in a dementia clinic by standard accepted clinical diagnostic criteria for AD of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA criteria). These individuals provided a total of 22 first-morning urine samples.
2. Normal: Seven cognitively normal individuals provided a total of 21 first-morning urine samples.

Previous studies have documented the sensitivity (90%) and specificity (91%) of the assay (28–30). The purpose of the present clinical study was to assess the longitudinal trend of individual levels (i.e., whether or not the NTP level in an individual with an initially elevated level remained elevated over time, and whether a normal remained normal over time).

RESULTS

Assay Optimization

All parameters in the assay were varied and studied in exhaustive permutations. The optimization included buffers (type, molarity, pH), blocking solutions and

TABLE 1. Analytical threshold*

Replicate number	54 μg/mL	43 μg/mL	23 μg/mL	10 μg/mL	8 μg/mL	5 μg/mL	2 μg/mL	1 μg/mL
1	0.297	0.467	0.762	1.265	1.756	1.891	2.036	2.036
2	0.321	0.487	0.752	1.332	1.689	1.854	1.979	2.045
3	0.352	0.497	0.797	1.279	1.787	1.798	1.956	1.997
4	0.322	0.459	0.793	1.257	1.812	1.834	2.036	2.054
5	0.334	0.521	0.775	1.331	1.697	1.882	1.954	2.010
6	0.342	0.524	0.769	1.267	1.726	1.797	1.899	1.997
7	0.367	0.486	0.812	1.354	1.656	1.697	1.964	2.102
8	0.316	0.447	0.765	1.387	1.777	1.795	1.997	2.004
9	0.359	0.467	0.769	1.269	1.694	1.775	2.010	1.978
10	0.316	0.497	0.745	1.337	1.756	1.759	2.023	1.979
11	0.321	0.467	0.797	1.321	1.726	1.899	2.100	2.100
12	0.336	0.489	0.771	1.298	1.663	1.912	1.989	2.098
13	0.317	0.459	0.769	1.336	1.804	1.798	2.098	2.079
14	0.345	0.479	0.821	1.279	1.632	1.880	1.976	2.142
15	0.352	0.497	0.768	1.369	1.757	1.789	2.012	2.065
16	0.317	0.521	0.797	1.297	1.687	1.812	2.036	1.997
17	0.326	0.516	0.826	1.339	1.664	1.798	2.019	1.987
18	0.356	0.497	0.832	1.365	1.716	1.854	1.997	2.036
19	0.322	0.508	0.779	1.267	1.731	1.865	1.978	2.067
20	0.349	0.522	0.803	1.349	1.812	1.821	2.024	2.098
Mean (OD)	0.333	0.490	0.785	1.315	1.727	1.826	2.004	2.044
SD	0.018	0.024	0.025	0.040	0.054	0.054	0.047	0.049
%CV	5.5	4.9	3.2	3.1	3.2	3.0	2.3	2.4

*The semilog standard curve is linear in the 0.1–1.4 OD range, indicating analytical threshold of 10 μg/mL. The limit of detection is 1.315 OD.

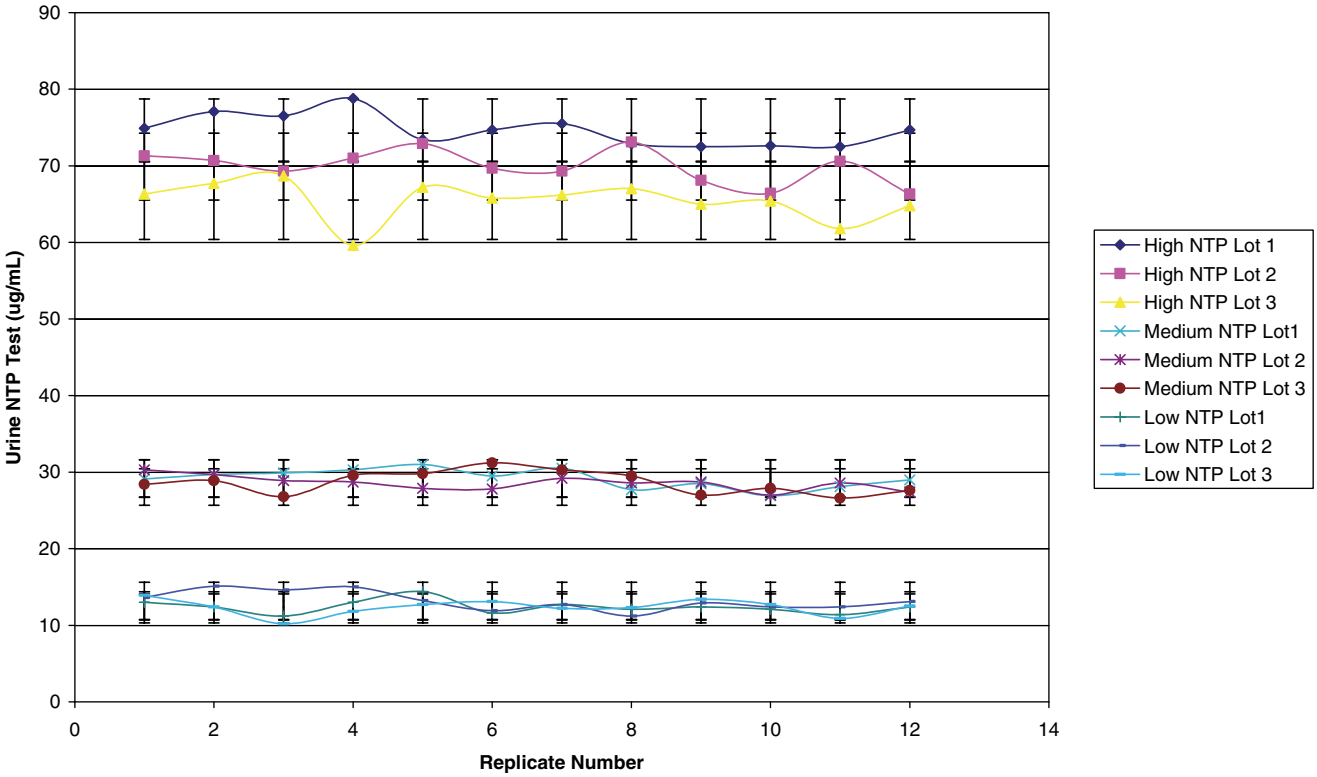

Fig. 2. Lot to lot variation of urine controls (108 replicates with CVs of 4.3–8.6%).

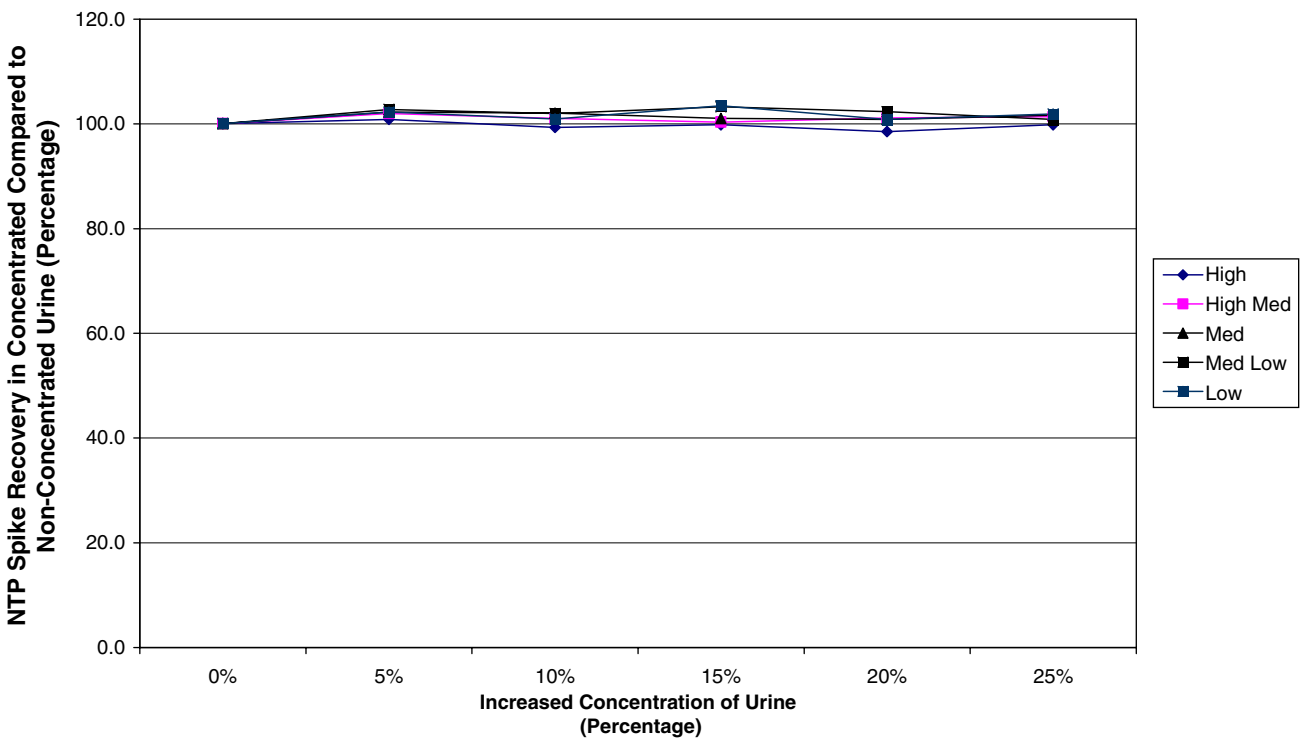

Fig. 3. Lack of effect of urine concentration on UNTP spike recovery.

agents, sample buffers and volume, wash solutions, enzyme buffers, enzyme concentrations, number of washes, temperature studies, and the duration of individual incubations. The goal of optimization was to have low background, high signal, and minimal noise while maintaining the relative speed, ease, and practicality of the assay as a reproducible routine test.

Linearity

The assay is linear in the working range (10–60 μg/mL) with $r^2 > 0.99$.

Analytical Threshold (Table 1)

Low-NTP urine was spiked to provide samples with urine neural thread protein (UNTP) concentrations of 54, 43, 23, 11, 8, 5, and 2 μg/mL. A total of 20 replicates of each were assayed. The semilog standard curve is linear in the 0.1–1.4 OD range. The limit of detection (at 405 nm) is OD 1.315 ± 0.8, corresponding to a threshold of 10 μg/mL.

Reproducibility

Precision Studies

A total of 720 replicates were assayed at four different clinical laboratory sites by four different trained laboratory personnel, on three different days each, consisting of high, medium, and low urines in 20 replicates each per day. The CVs varied from 2.3% to 7.1% (high-NTP urines), 1.5% to 8.5% (medium-NTP Urine), and 2.5% to 15% (low-NTP urine). Overall average CV was 6.2%. Lot to lot variation (Fig. 2): three lots of high-, medium-, and, low-NTP controls were tested in four replicates each for 3 days (108 replicates total). The CVs varied for 4.3–8.6%.

Recovery Studies

Low-NTP urine samples were spiked with known concentrations of NTP to 18.9, 23.9, 28.9, 33.9, and 38.9 μg/mL, and 20 replicates of each were assayed. The mean recovery from the 120 replicates was 105.5%.

Drug Interference Studies

Low-, medium-, and high-NTP urines were tested before and after spiking with the following drugs at 25 μg/mL concentration: acetaminophen, alprazolam, cephalexin, diltiazem, furosemide, Capoten, potassium, Fosamax, lanoxin, Lipitor, Losec, Pepcid, Vasotec, Zoloft, Adalat, atenolol, glyburide, hydrochlorothiazide, metoprolol, temazepam, amoxicillin, Ativan, Biaxin, Demerol, Indur, Norvasc, tetracycline, Synthroid, Prozac, flurazepam,

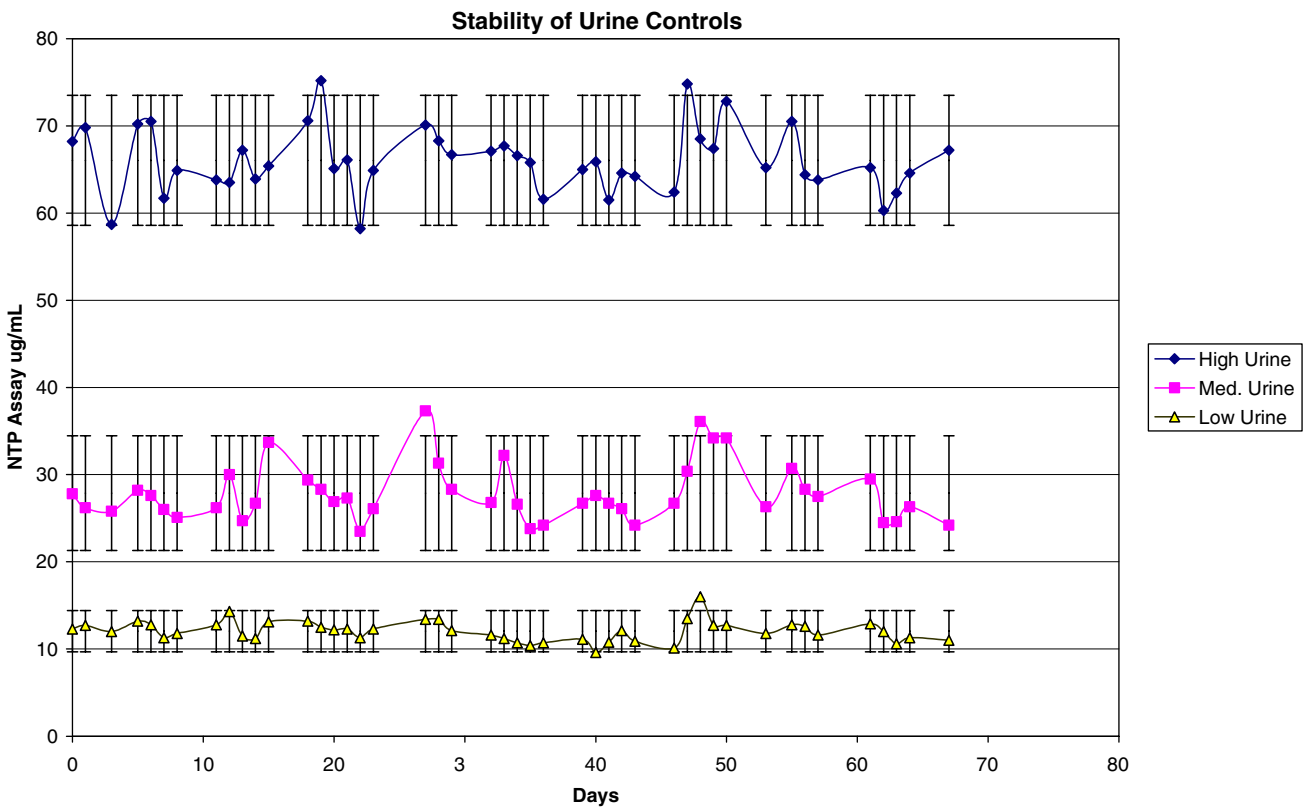

Fig. 4. Stability studies of urine controls showing stability >65 days.

ibuprofen, coumadin, metformin, and codeine phosphate. None of the spiked drugs had a significant effect on the NTP urine test values of any of the urine samples.

Other Interference Studies

Low-, medium-, and high-NTP urines were tested before and after spiking with the following substances: human gamma globulin (100 μg/mL), acid–1-glycoprotein (100 μg/mL), human serum albumin (100 μg/mL), bilirubin (20 μg/mL), fresh blood (250 red blood cells [RBC]/mL), hemolyzed blood (250 RBC/mL), gram negative bacteria, and lipids (cholesterol and lipoprotein; C-5555, Sigma). None of the above spiked substances had significant effect on the NTP urine test values of any of the urine samples.

Urine Concentration Studies (Fig. 3)

High-NTP urine was spiked into low-NTP urine, where the latter, before spiking, was concentrated at five different levels (5%, 10%, 15%, 20%, and 25%), and the spiked variably concentrated urines were then assayed (five levels of urine concentration for each of five levels of NTP concentration (95, 62.2, 29.4, 24.7, and 20 μg/mL). There was no significant effect of concentration of urine on the UNTP values.

Stability Studies (Fig. 4)

Stability studies indicate that refrigerated coated plates are stable for 8 weeks. Standard powder is stable for $\geq$6 months. Other kit components have stability of $\geq$1 year.

Freeze-Thaw of Processed Urine

High-, medium-, and low-NTP processed urine samples were assayed, frozen at –80°C, and then tested in 135 replicates on 45 different days up to 67 days. The CVs of the individual samples were 5.6% (high-NTP urine), 11.8% (medium-NTP urine), and 9.8% (low-NTP urine).

Clinical Study (Table 2)

All seven AD individuals had elevated NTP levels. A total of 15 repeated readings at intervals of 2 days to 2 years all remained elevated (there were no examples of AD individuals with an elevated level subsequently testing normal). The mean overall AD value was 44.3 (standard deviation [SD] 12.4). The AD individuals mean age was 83.2 (SD 23.7). One normal individual (male, age 78 years) had an initial value of $<$10 μg/mL, followed by a repeat value at 3 months' interval of 15.3 μg/mL. At an interval of 3 years, his NTP level was 29 μg/mL, and at 4.5 years it was 46 μg/mL. This individual (excluded from the mean value calculations above) was asymptomatic at the first sample, but had a clinical diagnosis of AD 3 years later.

Overall, the 43 clinical samples showed excellent correlation with the clinical diagnosis, and longitudinal stability. There were no cases of AD that reverted to normal NTP levels from initially elevated levels.

TABLE 2. Test–retest values over time in AD and control subjects

Subject number	Age	Sex	NTP (μg/mL)	Interval (I = initial)
1	41	F	13	I
			18.4	1 month
			16.4	2 months
2	79	F	22	I
			29.1	1 month
3	79	M	37.6	I
			43.5	1 month
4	101	F	42.4	I
			47.1	2 days
5	91	F	$>$60	I
			53.2	1 month
			56.3	1 month
			$>$60	2 months
			54.4	2 months
			$>$60	3 months
6	3	M	$<$10	I
			21.5	1 year
7	77	F	20.7	I
			10.1	1 year
			11	2 years
			19	2 years
8	81	M	22	I
			29.7	1 month
			46.	2 years
9	71	M	$>$60	I
			45	8 months
			39.4	8 months
10[a]	78	M	$<$10	I
			15.3	1 month
			29[a]	3 years
			46[a]	4.5 years
11	45	M	$<$10	I
			13.4	1 year
12	81	F	$>$60	I
			43.8	2 weeks
			30.9	3 months
			32.8	3 months
13	42	F	17.7	I
			14.9	2 months
15	38	M	11.3	I
			12.3	1 week
			11	4 months
			11.6	5 months

[a]Clinical diagnosis of AD 3 years after initial measurement. Patient asymptomatic at initial measurement.
F, female; M, male.

The NTP levels of the seven individuals in the study who were cognitively normal remained consistently in the normal range, with the single exception of the 78-year-old male whose NTP level increased in apparent tandem with his clinical evolution of new onset of AD.

DISCUSSION

The new assay described in this report detects NTP in the 10–60 μg/mL range, a 1,000-fold increase compared to earlier published polyclonal antibody NTP assay, which detected NTP at a maximum level of <100 ng, and which used recombinant protein standard material that was difficult to manufacture reliably (24,28–30). The newer, synthetic standard used in the present kit is stable (>6 months) and highly reproducible. The assay reliably measures UNTP from patients suspected of having the diagnosis of AD with a sensitivity of 90% and specificity of 91%, which is particularly useful for the nonspecialist, for which sensitivity and specificity on clinical grounds alone are in the 25–50% range (31–33). The values of individuals tested over time do not appear to fluctuate significantly with respect to the cutoff value, with positive values remaining elevated in subjects who were retested at intervals up to 2 years. The processing and assay steps do not require any overnight or lengthy incubations, and a single operator can perform large numbers of samples (estimated 2,000 data points per week). The precision studies demonstrate CVs on average of 6.2%. The competitive ELISA UNTP assay kit has significant potential to aid in the evaluation of therapeutics for AD. With an accurate noninvasive peripheral marker, therapeutic trials can be monitored more frequently and objectively. A technically simple and accurate noninvasive peripheral test such as the UNTP assay will have significant practical use in the routine clinical evaluation of elderly patients at risk for AD.

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