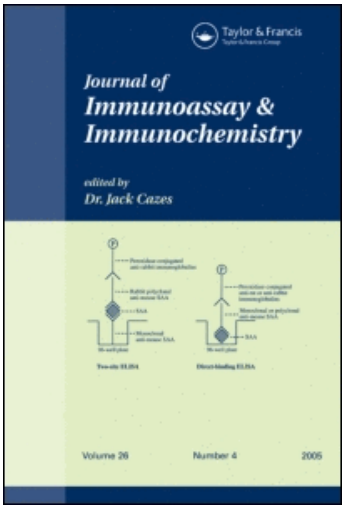

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Development of an ELISA Test for Determination of the Urinary Trypsin Inhibitor: Analytical Performance and Applications

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Abstract: Increased urinary excretion of urinary trypsin inhibitor (UTI) has been reported in various inflammatory conditions and in Alzheimer's subjects, but its diagnostic potential remains to be elucidated. A reliable and specific enzyme-linked immunosorbent assay (ELISA) test for the determination of the UTI in human urine was developed. This assay was performed using 96-well microtiter plates. The plate surface is coated with an anti-UTI polyclonal antibody, the urine sample was added in a dilution range, and the detection was achieved using the enzyme-conjugated antibody. The assay was quantified by the build-up of colored product upon the addition of the substrate. Recoveries were 93%, and the intra- and inter-assay CVs were 4.25% and 21%, respectively. The ELISA showed parallelism of standard and urine samples and no significant interference by the biological matrix. The usefulness of the assay has been demonstrated by applying it to urine samples from Alzheimer's

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disease patients, and comparing with negative controls. UTI urinary levels are significantly increased in Alzheimer's subjects.

Keywords: Urinary trypsin inhibitor, Enzyme-linked immunosorbent assay, Alzheimer, Quantitation, Analytical accuracy, Synthetic urine

INTRODUCTION

The urinary trypsin inhibitor (UTI), also known as bikunin, is a multipotent serine proteinase inhibitor belonging to the Kunitz inhibitors,[1] which can be found in biological fluids in free or combined form.[2] It is produced by proteolytic fragmentation of inter-α-trypsin inhibitor (ITI) a plasmatic inhibitor which is synthesized in the liver.[3] UTI is present in human[4] and is excreted in the urine of healthy humans within a range of several mg/day and is involved in many physiological and pathological processes.[5,6] UTI is rapidly released in the urinary flow after an infection; it is supposed to be an inflammation marker.[7] Owing to its antifibrinolytic activity, UTI appears to play an important role in the metabolism of tumor cells and its levels are increased in the urine of cancer bearing patients.[8–11] It has also been found as a component of the brain neuritic plaques of Alzheimer's disease patients[12] and UTI levels increase in the urine of the same subjects.[13] UTI-like inhibitors have been evidenced in neurons and in astrocytes; they seem to be synthesized as a response to stress conditions at cerebral level,[14] as it can be deduced by an increase of the immunoreactive substance in several brain regions, in particular, in the hyppocampus, determined by cerebral damages.[15] On the other hand, the possible involvement of this inhibitor in the aetiology of Alzheimer's disease is supported by an impairment of the proteolytic/antiproteolytic processes that causes the genesis of the neuritic plaques, the major biological hallmark of the disease.[16,17] In this paper, we report the analytical performance of an enzyme-linked immunosorbent assay (ELISA) test developed for the detection of UTI in human urine,[18–20] and the obtained results applied to elder subjects, both healthy and affected by Alzheimer's disease.

EXPERIMENTAL

Chemicals

Horseradish peroxidase (HRP), bovine serum albumin (BSA), and *ortho*-phenylenediamine (OPD) were obtained from Sigma chemical Co (USA). Polyclonal anti-human ITI antibodies were obtained from Dakopatts (Denmark) and utilized without further purification. Human UTI and polyclonal anti-human

UTI antibodies, raised in rabbits, were kindly supplied by Mochida pharmaceutical company (Japan). The 96-well microtiter plates used for immunosorbent assay (MaxiSorp F16) were purchased from Nunc A/S (USA). The phosphate buffer (PBS), pH 7.2, was prepared in concentrated solutions (76.50 g of NaCl, 7.24 g of Na_2HPO_4, and 2.10 g of KH_2PO_4 dissolved in 1 L of deionized water) to avoid bacteria contaminations and diluted 1 to 10 just before use. The washing buffer (WB) was prepared from PBS by slowly adding 0.05% of Tween-20. All other chemicals were of analytical grade.

Collection and Processing of Urine Samples

The urine samples utilized for the UTI-like inhibitor quantitation were collected in sterile containers in the morning, maintained at 4°C and treated within 4 hr after collection (see Results). The samples were obtained from voluntary donors clinically assessed at the Geriatric hospital of INRCA (Fermo, AP) and at the Neurology Unit of the Macerata Hospital. Fifty-eight subjects (age range: 65–80 years) were selected according to an inclusion protocol that expected a test of the cognitive functions, the absence of an inflammatory state (using opportune blood tests), and a negative anamnesis for neuropsichiatric diseases related to acute or chronic flogosis. All samples were collected after informed consent had been obtained from the patients.

AD was diagnosed according to the NINCDS-ADRDA criteria of the diagnostical statistical manual of mental disorders (DSM III revised) (American Psychiatric Association, 1980). Eighteen subjects were classified as affected by Alzheimer's disease, since the criteria reported by NINCDS-ADRDA work group were satisfied.[21]

The calibration test was performed using a standard matrix, called synthetic urine, with a chemical composition very close to that of a 24 hr sample of human urine and prepared according the following protocol: 0.200 L of water, 16.5 g/L of urea, 0.37 g/L of uric acid, 0.99 g/L of creatinine, 0.165 g/L of glycine, 0.7 g/L of K_2HPO_4, 2.25 g/L of NaCl, 0.5 g/L of $CaCl_2$, 0.5 g/L of $(NH_4)_2SO_4$, and 0.005 g/L of urobilin IX.[22]

The quantitation of the analyte was performed using a serial 32-fold dilution of a synthetic urine containing a fixed quantity of UTI. After dilution, the UTI's concentration ranged between 2×10^{-3} g/L to 0.5×10^{-8} g/L.

Protein Concentration Determination

Protein concentration was determined using the Bradford micromethod, utilizing BSA as standard.[22]

Urinary Creatinine Content

The creatinine content of the urine samples assayed with the ELISA test was determined using an enzymatic colorimetric test (Roche Diagnostic).[23]

Urinalysis

Prior to sample utilization, a complete urinalysis was carried out by standard and routinely accepted methods by clinical laboratories (Stick Combur test).[24]

Anti-ITI Antibodies Labelling

A portion of anti-ITI antibodies was labeled with HRP and the method was performed according to the Nakane and Kawaoi procedure.[25] The concentration of the immunoglobulin was adjusted to 8 g/L and the solution was frozen at −20 °C. This preparation has retained its activity for more than 1 year.

ELISA Test

The ELISA test developed for the detection of UTI in human urine conforms to the sandwich type and utilizes polyclonal anti-UTI antibodies (1.2 g/L) as the primary antibody. It was prepared according to the following steps.

a. *Coating with anti-UTI antibodies:* polystyrene 96-well microtiter plates (MaxiSorp F16) were coated with 100 μL/well anti-UTI (1.2 g/L) diluted 1/4000 in 0.1 mol/L sodium carbonate pH 9.0, (100 μL/well), overnight at 4°C.
b. *Blocking with 1% BSA in PBS:* after being washed three times with PBS + 0.05% Tween-20, the plate was blocked (200 μL/well) for 2 hr at room temperature with PBS containing 1% BSA.
c. *Antigen loading:* urine samples, serially diluted in PBS + 1% BSA (100 μL/well), were added to the coated well and incubated 1 hr at room temperature, in a humidified box.
d. *Anti-ITI-HRP antibodies loading:* after the plate had been washed for three times with PBS + 0.05% Tween-20, HRP-conjugated anti-ITI antibodies (8 g/L stock solution) diluted 1 : 500 in PBS + 1% BSA were added to each well (100 μL/well), and the plate was incubated 1 h at room temperature in a humidified box.
e. *OPD loading:* after another three washes with PBS + 0.05% Tween-20, 200 mL of the substrate solution, containing OPD (0.4 mg/mL initial

concentration) dissolved in 0.1 mol/L Na PBS and 0.1 mol/L citric acid pH 5.0, were dispensed in each well, adding 0.01% H_2O_2 immediately before the loading procedure.

f. *Reaction blocking:* the plate was incubated at room temperature for 5 min and the reaction was stopped by addition of 3 N sulfuric acid solution (50 μL/well).

g. *Reading and data analysis*: the plate was read on a spectrum microplate reader (Labsystems Multiskan Ascent, Finland) at a wavelength of 492 nm.

During the work-up conditions phase, urines from the same donor were used in order to find the best conditions.

Rationale

In ELISA determinations, the absorbance (Abs) is the experimental response measured at a given antigen quantity (Ag_{TOT}) added into the well. The experimental response is related to the antigen titer by a simple logistic Eq. (1)

$$\mathrm{Abs} = \mathrm{Abs}^{\infty} + \frac{\mathrm{Abs}^{0} - \mathrm{Abs}^{\infty}}{1 + \left(\mathrm{Ag}_{\mathrm{TOT}}/\mathrm{ED}_{50}\right)} \tag{1}$$

where Abs^{∞} and Abs^{0} are the absorbance asymptotic values at infinite dilution and saturating concentration of the antigen, respectively. ED_{50} is the quantity of antigen having an absorbance value Abs_{50} equal to:

$$\mathrm{Abs}_{50} = \mathrm{Abs}^{\infty} + \frac{\mathrm{Abs}^{0} - \mathrm{Abs}^{\infty}}{2}$$

The parameter n is related to the overall stoichiometry of the antigen–antibody recognition process.

In the case of a given initial antigen quantity Ag_{TOT} is diluted D-folds, the relevant equation is

$$\mathrm{Abs} = \mathrm{Abs}^{\infty} + \frac{\mathrm{Abs}^{0} - \mathrm{Abs}^{\infty}}{1 + \left(\left(\frac{\mathrm{Ag}_{\mathrm{TOT}}}{D}\right)/\mathrm{ED}_{50}\right)^{n}} \tag{2}$$

Of course, D will increase during dilution.

Usually, ELISA data are represented as Abs vs. log Ag plots. At any given antigen value, it is possible to calculate the derivative

$$m = \frac{\partial\ \mathrm{Abs}}{\partial\ \log \mathrm{Ag}} = \frac{1}{2.302}\frac{\partial\ \mathrm{Abs}}{\partial\ \ln\ \mathrm{Ag}}$$

Around the ED_{50} value, the logistic curve can be approximated to a linear function. In this case, the derivative m can be derived as

$$m = \frac{n}{4}\frac{\mathrm{Abs}^0 - \mathrm{Abs}^\infty}{2.302}$$

It is noteworthy that, around ED_{50} (i.e., in the linear zone), the value of m is related only to n and to the difference between the two absorbance asymptotes.

On the other hand, m represents the slope of the linear approximation

$$\mathrm{Abs} = m\ \log \mathrm{Ag} + q \tag{3}$$

If we consider the dilution of a sample in which a given quantity of antigen Ag_{TOT} is present, Eq. (3) can be rearranged as

$$\mathrm{Abs} = m\ \log\frac{\mathrm{Ag_{TOT}}}{D} + q = (m\ \log \mathrm{Ag_{TOT}} - m\ \log D) + q$$

or, in another way

$$\mathrm{Abs} = (m\ \log Ag_{TOT} + q) - m\ \log D$$

This latter equation demonstrates that in ELISA experiments, dilution of different samples (i.e., with different Ag_{TOT} quantities) can be analyzed using a straight parallel linear function if the antigen is around the ED_{50} value. The y-intercept $q^* = (m \log \mathrm{Ag_{TOT}} + q)$ is the only quantity related to the antigen present in the sample, while the slope will be unaffected by the antigen titer as long as the assay is carried out with the same antibody lot.

RESULTS AND DISCUSSION

Pre-Analytical Phase

The urine samples were collected in the morning and maintained at 4°C. UTI determinations were made within 4 hr. The exceeding sample was stocked and maintained at −20°C. The freezing procedure did not affect the results of the analyte determination (data not shown).

Anti-UTI Antibodies Dilution

A dilution of 1 : 4000 of the anti-UTI antibodies (1.2 g/L) for the plate coating was used (Figure 1). The chosen dilution allowed us to have a maximal value of absorbance at 492 nm at a constant value of antigen concentration, minimizing the "hook effect" determined by high antibody concentrations (data not shown).[26]

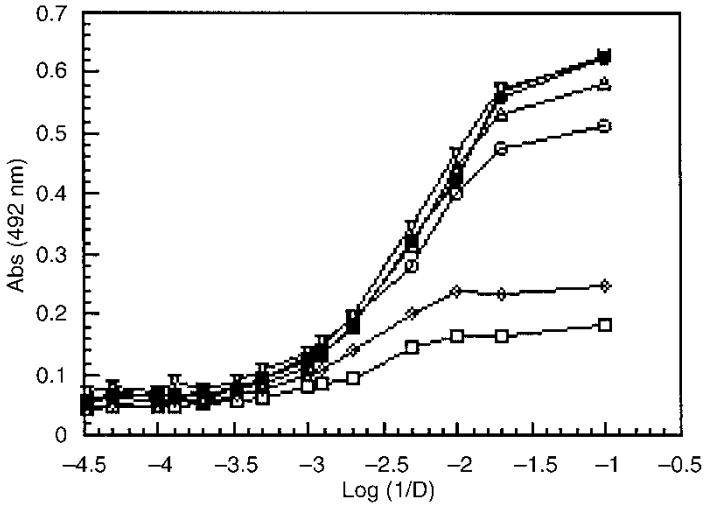

Figure 1. ELISA determinations of UTI solutions, at increasing dilution from 1/10 to 1/32,000. The experiment repeated changing the polyclonal antibody anti-UTI concentration (1.2 g/L): (□), 1/40,000; (◇), 1/30,000; (○), 1/10,000; (*), 1/5,000; (▽), 1/4,000; (△) 1/3,000; (•) 1/500.

Calibration Curve

Figure 2 shows a calibration curve for a synthetic urine solution containing a fixed amount of UTI, obtained through a serial 32-fold dilution (see Experimental). After dilution, UTI concentrations ranged from 2×10^{-3} g/L to 0.5×10^{-8} g/L. The calibration curve reported is the result of five replicates. The parameters (n, ED_{50}, Abs^{∞}, and Abs^{0}) (see Rationale) of the curve were independently calculated. The data obtained were then analyzed according to the logistic Eq. (2), obtaining a second set of parameters listed as follows: Abs^{0}, 2.158 $\pm$ 0.022; Abs^{∞}, 0.106 $\pm$ 0.013; $ED_{50\ (pg/100\,mL)}$, 1.584 $\pm$ 0.067; n, 1.158 $\pm$ 0.052.

The five calibration curves did not significantly differ to each other (95% confidence level) when analyzed by F-test;[27] therefore, they belonged to the same statistical population. Consequently the parameters reported earlier characterize the calibration curve and they can be used for UTI determinations on the urine samples.

Antigen Determination in Untreated Human Urine

A urine sample was serially diluted from 1 : 2 to 1 : 30,000. Utilizing the logistic Eq. (2) and the same parameters of the standard calibration curve

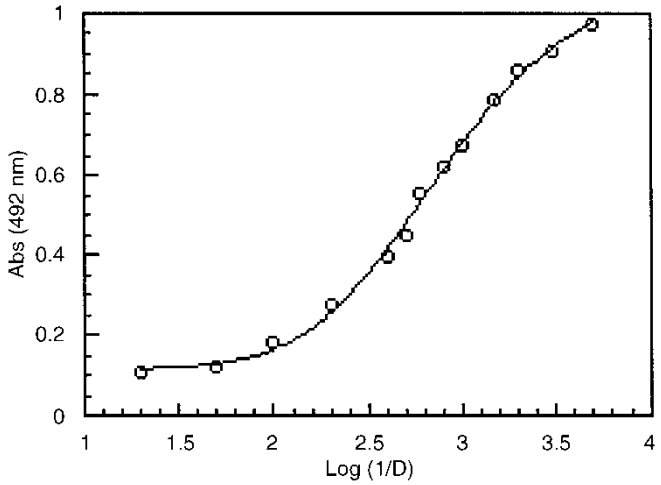

Figure 2. Calibration curve of a solution of synthetic urine spiked with fixed quantity of UTI (from 2×10^{-3} g/L to 0.5×10^{-8} g/L) obtained through a serial 32-fold dilution (see Experimental).

(see Results), it is possible to quantitate the total analyte (Ag_{TOT}) present in the sample. The mean value of UTI of $2.06 (\pm 0.16)$ mg/L in urine was obtained from five separate determinations.

Detection Limit

The minimum quantity of UTI that can be detected with this ELISA test is 4 pg/L $(Ag_{TOT})_{min}$. By comparison of the frequency distribution ($n = 96$) of a negative sample with a very dilute sample ($D = 30{,}000$) of untreated urine and utilizing the equation

$$t_{oss} = \frac{Abs_{1/D} - Abs_{\infty}}{SD_m/\sqrt{n}}$$

where SD_m is the standard deviation of the mean, we obtained (95% confidence level) that the absorbance produced by the analyte present in a urine sample diluted 1 : 30,000 is significantly above the buffer control (4×10^{-7} g/L).

The detection limit was also calculated, applying the Student-*t* test to the frequency distribution of a UTI negative sample for a limited number of repeats ($n = 4$) and utilizing the standard Equation:[28]

$$Abs_{min} = Abs^{\infty} + \frac{t_{0.95} \times SD}{\sqrt{n}}$$

Then, the Abs_{min} value obtained is substituted in Eq. (1), reported in Rationale; it is consistent with the calculated $(Ag_{TOT})_{min}$.

Imprecision, Recoveries, and Dilution Analysis

The intra-assay and inter-assay coefficients of variation were 4.25% and 21%, respectively. They were determined by performing 16 replicates of four dilutions (1 : 1000, 1 : 800, 1 : 500, 1 : 200) with the same urine sample. The procedure was repeated for 10 days, consecutively (data not shown).

The ELISA test developed is characterized by high specificity, as it comes out by the parallelism test[29,30] performed either with serial dilutions of synthetic urine spiked with increasing concentrations of UTI (1×10^{-4} g/L, 10×10^{-4} g/L, and 50×10^{-4} g/L) or serial dilutions of urine from healthy, as well as ill, subjects (Figure 3). The results obtained indicate that the antigen–antibody reaction is not significantly influenced by the biological matrix or by its components.

When urine samples and synthetic urines containing 2×10^{-3} g UTI/L were serially diluted and assayed, each sample gave results close to linearity ($r = 0.998$–0.982; Figure 4), confirming parallelism between the calibrators and urine samples.

The range of linearity for this curve is 5×10^{-6}–2.5×10^{-6} g/L. The recovery of the test is 92.79% $\pm$ 19.3% and it was obtained when seven different concentrations of UTI (0, 1×10^{-6} g/L, 2×10^{-6} g/L, 4×10^{-6} g/L, 5×10^{-6} g/L, 6×10^{-6} g/L, 8×10^{-6} g/L) were added to 16-fold serially diluted control urines (Figure 5).

The internal quality control of the test was also determined using a 1% serum albumin solution spiked with a known amount of analyte (UTI, 0.0333×10^{-6} g/L) and it indicated that the accuracy of UTI determinations is not affected by systematic errors (Figure 6).

UTI Levels in Elder Subjects

The ELISA test was utilized for the determination of the UTI urinary levels in 58 subjects. The ELISA test was used to study samples from 40 control patients and 18 patients diagnosed as Alzheimer. They were selected according to the inclusion criteria previously reported and classified as Alzheimer or controls on the basis of dementia diagnosis (see Experimental). For each subject, a dilution curve was obtained from which the mean value of UTI was calculated according to the logistic Eq. (2) (see Rationale). The effect of the total proteinuria, on the analyte concentration was taken into account, normalizing the UTI value for the creatinine content of the sample. Serial dilutions of a UTI standard solution (0.0333×10^{-3} g/L) were included in

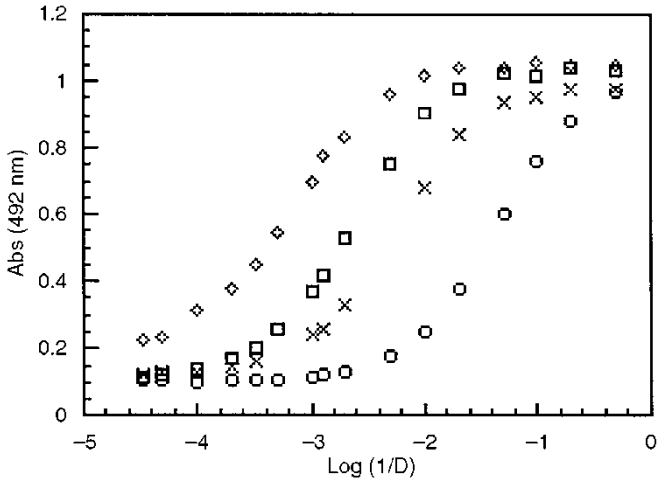

Figure 3. Serial dilutions of synthetic urine spiked with increased concentrations of UTI: (○), 1×10^{-4} g/L; (□), 10×10^{-4} g/L; (◇), 50×10^{-4} g/L; and (x) serial dilutions of human urine.

each plate. The results obtained for the 58 subjects are reported in Figure 7, where it is evident that the UTI urinary levels are significantly increased in ill subjects, confirming the previously published observations.[13] However, the analyte values are affected by a high standard deviation, indicating a

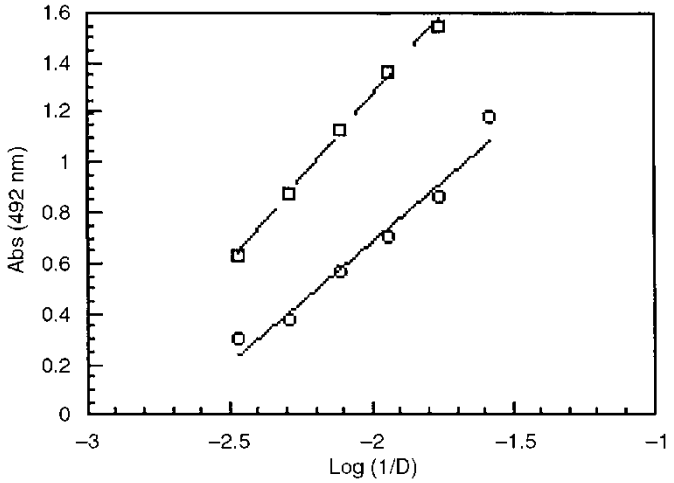

Figure 4. Linearity of dilution curves for urine samples. Data are based on serial dilution of normal urine samples and synthetic urine spiked with 2×10^{-3} g/L UTI (□).

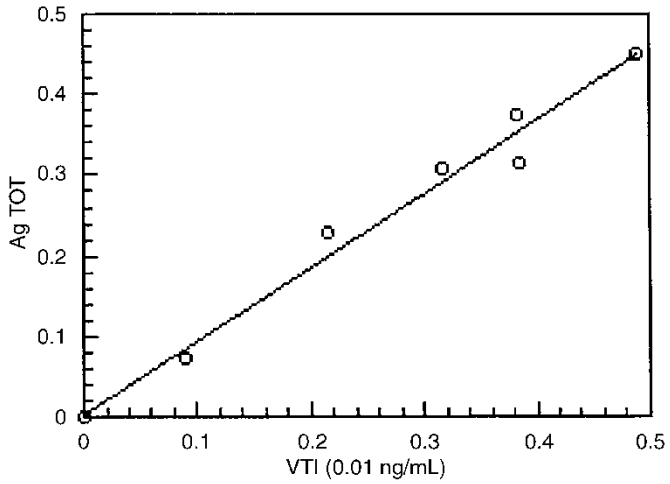

Figure 5. The recovery test obtained when seven different concentrations of 0, 1×10^{-6} g/L, 2×10^{-6} g/L, 4×10^{-6} g/L, 5×10^{-6} g/L, 6×10^{-6} g/L, 8×10^{-6} g/L were added to 16-fold serially diluted control urine.

considerable biological variability of the screened population. Anyway, the examined subjects are distinguishable by two distinct populations (healthy and ill subjects) with a good confidence level (70%) using the ANOVA test.[28]

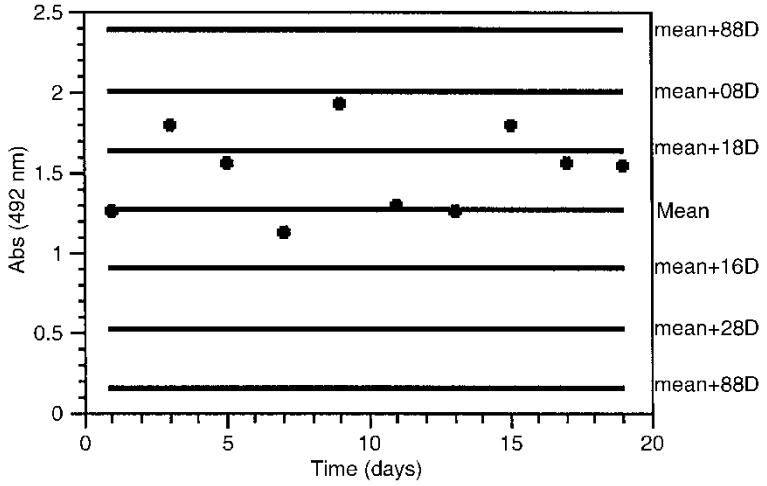

Figure 6. Control chart obtained with a fixed quantity of UTI (0.0333×10^{-3} g/L in 1% serum albumin solution) added in the analytical ELISA test.

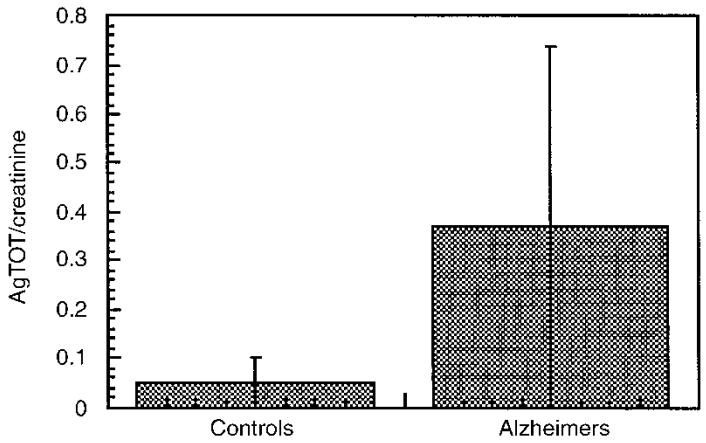

Figure 7. Mean concentration and standard deviation of the UTI levels on Alzheimer's patients and control's population normalized to urine creatinine content.

CONCLUSIONS

The study presented here was undertaken with the aim of achieving a simple and reliable method for the quantitative determination of the UTI, a serine protease inhibitor which is involved in many human diseases, including malignancies and neurodegenerative disorders. Since, in a previous paper, we reported that urines of Alzheimer's disease patients showed increased levels of UTI, we thought to apply the test to elder subjects in order to verify if it could have diagnostic significance. The data reported indicate that, from an analytical point of view, the test shows analytical performance characterized by high levels of sensitivity, analytical specificity, and accuracy. In addition, it can be applied to untreated human urines and it is rapid, simple, safe, and relatively inexpensive, i.e., very important characteristics for the application of the test to screening studies. Even though the previously reported observations of increased levels of UTI in the urine of Alzheimer's subjects have been confirmed, the diagnostic significance of the test needs to be further investigated. In fact, the number of patients to be screened should be increased in order to assess the diagnostic sensitivity of this ELISA determination to be added to the Alzheimer's disease diagnosis protocol.

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