Effects of Processing and Storage Conditions on Amyloid β (1–42) and Tau Concentrations in Cerebrospinal Fluid: Implications for Use in Clinical Practice

Niki S.M. Schoonenboom,1,2† Cees Mulder,2† Hugo Vanderstichele,3 Evert-Jan Van Elk,2 Astrid Kok,2 Gerard J. Van Kamp,2 Philip Scheltens,1 and Marinus A. Blankenstein2

Background: Reported concentrations of amyloid β (1–42) (Aβ42) and tau in cerebrospinal fluid (CSF) differ among reports. We investigated the effects of storage temperature, repeated freeze/thaw cycles, and centrifugation on the concentrations of Aβ42 and tau in CSF.

Methods: Stability of samples stored at −80 °C was determined by use of an accelerated stability testing protocol according to the Arrhenius equation. Aβ42 and tau concentrations were measured in CSF samples stored at 4, 18, 37, and −80 °C. Relative CSF concentrations (%) of the biomarkers after one freeze/thaw cycle were compared with those after two, three, four, five, and six freeze/thaw cycles. In addition, relative Aβ42 and tau concentrations in samples not centrifuged were compared with samples centrifuged after 1, 4, 48, and 72 h.

Results: Aβ42 and tau concentrations were stable in CSF when stored for a long period at −80 °C. CSF Aβ42 decreased by 20% during the first 2 days at 4, 18, and 37 °C compared with −80 °C. CSF tau decreased after storage for 12 days at 37 °C. After three freeze/thaw cycles, CSF Aβ42 decreased 20%. CSF tau was stable during six freeze/thaw cycles. Centrifugation did not influence the biomarker concentrations.

Conclusions: Repeated freeze/thaw cycles and storage at 4, 18, and 37 °C influence the quantitative result of the Aβ42 test. Preferably, samples should be stored at −80 °C immediately after collection.

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In the last decade, many studies have set out to find an appropriate biochemical marker for the diagnosis of Alzheimer disease (AD). Several authors have shown that the sensitivity and specificity of amyloid β (1–42) (Aβ42) and total tau in cerebrospinal fluid (CSF) are high when comparing AD patients with controls (1, 2). However, when comparing AD with other types of dementia (3), overlap occurs, hampering clinical utility. Ideally, the diagnostic value of biomarkers needs to be validated in neuropathologically confirmed cases, but most studies use clinical criteria as the reference standard, with the risk of circular reasoning. Furthermore, use of the markers in clinical practice still needs to be established, as most studies have been carried out in research settings with selected patient samples (4).

A recent metaanalysis (5) demonstrated considerable variability in absolute concentrations of both markers among centers, even when using the same commercial assay. This variability could also be attributable to differences in patients or to a difference in processing and storage methods among centers.

Few published studies have investigated which factors produce a major influence on the quantitative outcome of the INNOTEST™ β-amyloid (1–42) ELISA (6, 7). An

1 Alzheimer Center and Department of Neurology, and 2 Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands.
2 Innogenetics NV, Ghent, Belgium.
3 Both authors equally contributed to this work.
4 Address correspondence to this author at: Departments of Neurology and Clinical Chemistry, VU University Medical Center, Postbus 7057, 1081 HV Amsterdam, The Netherlands. Fax 31-204440715; e-mail niki.schoonenboom@vumc.nl

Received July 5, 2004; accepted October 11, 2004.
Previously published online at DOI: 10.1373/clinchem.2004.039735

Nonstandard abbreviations: AD, Alzheimer disease; Aβ42, amyloid β (1–42); CSF, cerebrospinal fluid; MCI, mild cognitive impairment; FTLD, frontotemporal lobar degeneration; and MD, mixed-type dementia.
important confounding factor is the tendency of both Aβ42 and tau to adhere to glass or hard plastic tubes (4), reducing the concentration. Furthermore, repeated freeze/thaw cycles seem to play a role in the decrease of CSF Aβ42, although different methods have been used to investigate this phenomenon. One study (6) showed a large decrease in CSF Aβ42 between the first and second freeze/thaw cycles, whereas no difference was found between Aβ42 concentrations in fresh CSF and CSF that had been frozen and thawed once (7). No studies have been published regarding the stability of both Aβ and tau in CSF when stored frozen at −20 or −80 °C for many years. Knowing sample stability at freezing temperatures is especially important for longitudinal studies in which samples are stored for long periods and analyzed simultaneously with samples stored for short periods to minimize interassay variability.

In this study, we sought to answer the following questions: What are the stabilities of Aβ42 and tau in CSF samples stored at −80 °C for several years? What are the stabilities of Aβ42 and tau in samples stored at 4, 18 (room temperature), and 37 °C up to 3 weeks (to investigate the effect of mailing)? What is the effect of repeated freeze/thaw cycles on Aβ42 and tau concentrations in CSF? What is the effect of centrifugation? Awareness of preanalytical factors that may influence the concentrations of the markers could improve collaboration with other neurologic research centers or memory clinics and provide more reliable results. Our final aim was to formulate standardized conditions, which will be crucial when the use of Aβ42 and tau becomes standard practice for the (early) diagnosis of AD.

**Materials and Methods**

**Participants**

Twenty-three individuals provided CSF for the entire study: 3 AD patients, 5 patients with mild cognitive impairment (MCI), 5 patients with frontotemporal lobar degeneration (FTLD), 1 patient with mixed-type dementia (MD), and 9 controls with no dementia. All individuals gave informed consent to participate in the study. Four individuals entered the accelerated stability testing protocol. For the analysis of tau in this experiment, one sample was excluded because the results were higher than the values for the highest calibrator. Two of the four individuals whose CSF was used in the accelerated stability testing protocol also participated in the freeze/thaw experiment. In addition, 13 other individuals provided CSF for the freeze/thaw experiments: 5 for the comparison of unfrozen CSF with CSF frozen and thawed once, plus 8 for the comparison of samples frozen and thawed once with samples subjected to several freeze/thaw cycles. Six individuals provided CSF for the centrifugation experiment, including one who provided an additional hemolytic CSF specimen, which was not centrifuged and was compared with the baseline centrifuged specimen.

**Lumbar Puncture**

CSF was obtained by lumbar puncture in the L3/L4 or L4/L5 intervertebral space, using a 25-gauge needle, and was collected in 12-mL polypropylene tubes. A small amount of CSF was used for routine analysis, including total cells, total protein, and erythrocytes. Therefore, CSF samples were centrifuged as soon as possible (with a maximum of 2 h after collection) at 2100g for 10 min at 4 °C. CSF samples were kept at room temperature until centrifugation. After centrifugation, CSF was pipetted into polypropylene tubes in 0.11-, 0.2-, or 0.5-mL aliquots, depending on the experiment for which the CSF was to be used.

**Accelerated Stability Testing Protocol**

To study the stability at −80 °C, we used an accelerated stability testing protocol based on the principle of the Arrhenius equation (8), which describes a linear relationship between the logarithm of the reaction rate constant (e.g., the degradation rate) and the inverse of the absolute temperature. We used the results from samples stored at three temperatures, 4, 18, and 37 °C, to calculate the rate constant.

The principle and calculations for this protocol applying the Arrhenius method are given in the Appendix at the end of this article.

**Participants.** Two patients and two controls participated in the accelerated stability experiment. One patient was a 73-year-old male with MD, and the other a 54-year-old female with probable AD according to the clinical criteria (9). The controls were two spouses, without dementia, of patients: a 77-year-old male and a 58-year-old female.

**Samples.** After centrifugation, samples were divided into 0.5- and 0.2-mL aliquots. The 0.5-mL aliquot from each patient was stored immediately at −80 °C (193 K) to determine the baseline values for Aβ42 and tau. The other thirty 0.2-mL aliquots from each patient were stored at 4 °C (277 K), room temperature [18 °C (291 K)], and 37 °C (310 K), 10 tubes at each temperature. After 1, 2, and 3 days and up to 22 days, one tube stored at each of the three temperatures was removed and frozen at −80 °C until analysis. All 30 samples from each patient were thawed and analyzed, in duplicate, simultaneously in one run.

**Freeze/Thaw Cycles**

To compare unfrozen CSF with CSF frozen and thawed once, we stored, in polypropylene tubes, two 0.2-mL aliquots of CSF from five individuals (three with MCI and two with FTLD) for 2 days at 4 or −80 °C until analysis. The concentrations of Aβ42 and tau in the aliquots that had not been frozen and thawed (stored at 4 °C) were compared with the concentrations in the aliquots that have been thawed once (stored at −80 °C). All aliquots were tested in duplicate.
Because most samples are stored at −80 °C until analysis, the best way to simulate daily practice is to compare samples subjected to one freeze/thaw cycle with samples that have undergone several freeze/thaw cycles. Therefore, CSF from 10 individuals (1 patient with MD, 3 patients with FTLD, 1 patient with MCI, and 5 controls without dementia) was centrifuged and aliquoted into six 0.11-mL portions. One tube from each patient was kept at −80 °C until analysis, and the concentrations of Aβ42 and tau in this aliquot were used as the baseline values (100%). The other five tubes from each patient were stored at −80 °C and thawed two, three, four, five, or six times at room temperature for 2 h and stored again at −80 °C until analysis. The relative Aβ42 and tau concentrations (%) in the samples from the 10 patients were compared with the baseline value (100%) and plotted against the number of freeze/thaw cycles.

INFLUENCE OF TIME TO CENTRIFUGATION
CSF from five patients (two with AD, one with MCI, and two controls without dementia) was aliquoted into five 0.5-mL polypropylene tubes. Tube 1 was centrifuged at 2100g for 10 min at 4 °C within 2 h after CSF collection and then stored immediately at −80 °C. The concentrations of Aβ42 and tau measured in tube 1 were used as baseline values. Tubes 2, 3, and 4 were stored at 4 °C and centrifuged after 4, 48, and 72 h. After centrifugation, the supernatant was pipetted into polypropylene tubes and stored frozen for a maximum of 1 month until analysis. Tubes 5 were not centrifuged at all and were kept for 4 days at 4 °C before storage at freezing temperature. Relative Aβ42 and tau concentrations (%) in samples not centrifuged were compared with those in samples centrifuged after 1, 4, 48, and 72 h. In addition, we compared a hemolytic CSF sample (28 800 erythrocytes/μL, which is equivalent to ~0.5% whole-blood contamination), obtained after a traumatic lumbar puncture from a patient with MCI, that had not been centrifuged but had been stored at −80 °C with a sample from the same patient centrifuged within 2 h and stored at 4 °C until analysis.

ANALYSIS OF Aβ42 AND TAU
Aβ42 concentrations for all experiments were determined with the INNOTEST β-amyloid (1–42) sandwich ELISA (Innogenetics). Monoclonal antibody 21F12, which is highly specific for the COOH terminus of the Aβ42 peptide, was used as capture antibody, and the biotinylated monoclonal antibody 3D6, specific for the NH2 terminus, was used as detector antibody (6). For tau quantification, we used the INNOTEST hTau Antigen sandwich ELISA (Innogenetics), which is constructed to measure both total tau and phosphorylated tau with monoclonal antibody AT120 as capture antibody and HT7 and BT2 as detection antibodies (10). For the stability experiment, performed at Innogenetics, the mean intraassay CVs were calculated from the differences between duplicate measurements. The mean CVs for Aβ42 were 6.2% at Aβ42 concentrations ≤500 ng/L (n = 61) and 7.2% at Aβ42 concentrations >500 ng/L (n = 59). For tau, the mean intraassay CVs were 8.7% at tau concentrations ≤300 ng/L (n = 61) and 13% at tau concentrations >300 ng/L (n = 26). The mean CVs at the VU Medical Center laboratory were calculated based on the SD for the difference between duplicate measurements (SD × 100/mean) of a total of 60 routine samples. For Aβ42, the mean CV was 4.0% at concentrations in the low range (125–300 ng/L), 2.9% at concentrations in the middle range (600–800 ng/L), and 3.4% at concentrations in the high range (1000–2000 ng/L). For tau, the CVs were 6.5% at concentrations in the low range (75–200 ng/L), 4.7% at concentrations in the middle range (500–700 ng/L), and 4.6% at concentrations in the high range (900–1200 ng/L). The mean interassay CVs for three different pools, evaluated in advance and tested in the stability, centrifugation, and freeze/thaw experiments, were 12% for Aβ42 (n = 7) and 8.1% for tau (n = 7). Mean recoveries from four samples to which 1:1 dilutions of the highest calibrators for Aβ42 or tau were added were 77% (range, 73–81%) for Aβ42 and 109% (range, 108–112%) for tau.

RESULTS
STABILITY AT −80 °C ACCORDING TO THE ARRHENIUS EQUATION
The relative concentrations of Aβ42 and tau in CSF from the four and three patients, assayed on consecutive days, were calculated at 4, 18, and 37 °C, with the baseline value (−80 °C) set at 100%. We plotted the relative concentrations (%) vs the days of heat stress and calculated the rate constants at each investigated temperature [kT; Table 1]. The calculated kT values, determined at the three temperatures, were not different from zero, indicating that Aβ42 and tau are stable in CSF when stored at −80 °C.

STABILITY DURING MAILING CONDITIONS
When we plotted the mean CSF Aβ42 values for samples from four patients stored at the three different temperatures against time, the protein concentrations were highest at baseline (−80 °C), and during the first 2 days, the concentrations decreased by ~20% in samples stored at 4, 18, and 37 °C (Fig. 1). Thereafter, the concentration of Aβ42 in CSF remained relatively stable up to 22 days, although we observed considerable variability between aliquots from the same individual. Plots for tau showed

<table>
<thead>
<tr>
<th>Temperature, K</th>
<th>Aβ42</th>
<th>Tau</th>
</tr>
</thead>
<tbody>
<tr>
<td>277</td>
<td>−0.0043 (0.04)</td>
<td>0.0018 (0.03)</td>
</tr>
<tr>
<td>291</td>
<td>−0.0032 (0.03)</td>
<td>−0.0016 (0.03)</td>
</tr>
<tr>
<td>310</td>
<td>0.050 (0.03)</td>
<td>−0.039 (0.03)</td>
</tr>
</tbody>
</table>

* Percentages of remaining measurable protein were plotted vs days of heat stress at each temperature. Mean (SD) kT values were determined for the three temperatures as the slope of the best-fit line.
that the protein was stable in CSF at 4 and 18 °C, whereas the concentration decreased at 37 °C after ~12 days (Fig. 2).

**FREEZE/THAW CYCLES**

We found no difference between the concentrations of Aβ42 and tau in CSF that had not been thawed and CSF that had undergone one freeze/thaw cycle (visualized for Aβ42 in Fig. 3A). The mean (SD) relative concentrations (%) of Aβ42 vs the number of freeze/thaw cycles are shown in Fig. 3B. The Aβ42 concentration decreased by 20% after three freeze/thaw cycles, after which the concentration remained relatively unchanged, at 80% of the baseline concentration during six freeze/thaw cycles. The change in concentration varied among individuals, ranging from no change in the samples from one patient to a large decrease in the samples from another. We could not demonstrate a difference between samples with high (≥550 ng/L; n = 5) and low concentrations (<550 ng/L; n = 5) of Aβ42. The mean (SD) relative concentrations (%) of tau vs the number of freeze/thaw cycles are shown in Fig. 3C. We observed no change in tau concentrations during the six freeze/thaw cycles.

**INFLUENCE OF TIME TO CENTRIFUGATION**

We found no difference between Aβ42 and tau concentrations in CSF samples that were stored at 4 °C and centrifuged after 1, 4, 48, or 72 h. Furthermore, there was no difference between the concentrations of these markers in samples stored frozen after centrifugation and samples that were not centrifuged and were stored for 4 days at 4 °C. In addition, we found no significant difference in Aβ42 and tau concentrations in the hemolytic sample not centrifuged and stored at −80 °C (Aβ42 = 662 ng/L; tau = 232 ng/L) and the sample centrifuged and stored at 4 °C (Aβ42 = 596 ng/L; tau = 230 ng/L).

**Discussion**

Using the Arrhenius approach, we showed that Aβ42 and tau concentrations are stable in CSF samples frozen immediately and stored for a longer period at −80 °C. In addition, the concentration of Aβ42 in CSF decreased by ~20% during the first 2 days when stored at 4, 18, and 37 °C and then remained constant up to 22 days although with considerable variability among aliquots from the same individual. Tau concentrations in CSF remained stable at 4 and 18 °C, but showed a decrease after 12 days when stored at 37 °C. After three freeze/thaw cycles, the concentration of Aβ42 in CSF decreased by 20%, whereas tau remained stable during six freeze/thaw cycles. Centrifugation did not influence the results for either biomarker.

To the best of our knowledge, the stabilities of Aβ42 and tau in CSF samples stored at −80 °C for many years had not been systematically investigated previously. Only two studies concluded that Aβ42 (2, 5) and tau (5) remained stable in CSF when stored >6 months at −70 or −80 °C. The first study showed that the correlation between Aβ42 measured at different times during 1 year and reanalysis at one time was high (r = 0.96) (2). Unfortunately, the slope of the line is not mentioned in that study, and this could give more accurate information about degradation of the protein. The second study found no relationship between CSF Aβ42 or tau concentrations and shelf life (5). In our study we investigated the long-term stability at −80 °C with an accelerated stability testing protocol according to the Arrhenius method. We found no significant decrease in Aβ42 or tau concentrations at 4, 18, and 37 °C except for tau at 37 °C, and then only after 12 days. The degradation constant, therefore, was not different from zero, and no Arrhenius plot or projected stability time could be calculated. From this we conclude that both proteins are very stable in CSF and that samples can be stored for a very long period at −80 °C. However, a real-time stability experiment performed in the future is needed to confirm our data.
The stabilities of various forms of Aβ in CSF samples stored at different temperatures have been described in two studies (11, 12) that measured the proteins with in-house ELISAs. In the first study (11), the immunoreactivity of CSF Aβ40 and Aβ42 decreased by 8% and 10% when kept for 24 h at 20 °C, but remained stable the first 24 h at 4 °C. In the second study (12), CSF total Aβ concentrations were measured and found to be unstable if samples were stored at 20 °C, 4 °C, and room temperature, with the largest decrease during the first day and plateauing after the third day. Although different assay formats were used, measuring different types of Aβ, the abovementioned findings support our results of a decrease in Aβ42 concentrations in CSF during the first 2 days for samples stored at 4, 18, and 37 °C compared with storage at −80 °C. Although incubation at higher temperatures could have an effect on the binding capacity of Aβ42 (6, 13, 14), storage of CSF at different temperatures does not seem not to affect the Aβ42 concentration (6), which is supported by our findings showing comparable Aβ42 concentrations in CSF samples stored at 4 or 37 °C. There was a difference in Aβ42 concentration only between samples stored at −80 °C immediately after collection or stored at higher temperatures after collection. An interesting finding in one study (11) was that the antibody-binding capacity of synthetic Aβ42 was lower in CSF than in H2O. In addition, Aβ42 concentrations were lower in artificial CSF with physiologic concentrations of albumin than without albumin. An explanation for this finding could be that binding of Aβ42 to albumin masked the epitope recognized by Aβ42-specific antibodies (15, 16). This binding of Aβ42 to other proteins might also cause the low recovery rate of the Aβ42 ELISA, although Vanderstichele et al. (6) and others could not find interference with Aβ by other proteins, including albumin (17). However, interference experiments are largely dependent on the protocol being used or on whether preincubation is needed. Furthermore, results are also dependent on which medium is used, e.g., artificial CSF, human CSF, or another medium such as sample diluent (6). The difference in Aβ42 concentrations between samples stored at −80 °C and at higher temperatures could also be the result of binding of Aβ42 to other proteins, but conformational changes, aggregation (18), or degradation
heat-resistant filaments, consisting of a portion of tau, is protease- and temperature. The pathologic core protein of paired helical filaments has been demonstrated to increase at temperatures above 30 °C. The nature of tau in CSF is not well understood, but the aggregated pathologic form released in the CSF, probably as a result of degradation in the brain, suggesting that tau is truncated when reassembled further in future multicenter studies.

Our finding of decreased concentrations of Aβ42 in CSF after repeated freeze/thaw cycles is in agreement with the outcomes of several other studies (6, 11, 12) and stresses again the importance of avoiding freeze/thaw cycles to minimize the risk of falsely low Aβ42 values. The decrease in Aβ42 in CSF after repeated freeze/thaw cycles might also be explained by the same physicochemical mechanisms, i.e., conformational changes in the fibrillar β-sheeted Aβ42 protein or masking of the epitope by binding to other proteins, that led to a decrease in Aβ42 concentrations during the first 2 days in CSF samples stored at 4 °C. This is sustained by our finding of comparable CSF Aβ42 concentrations in samples that had not been subjected to a freeze/thaw cycle (stored for 2 days at 4 °C) vs samples that had been subjected to one freeze/thaw cycle.

Tau protein is considered to be very stable, and repeated freeze/thaw cycles do not seem to influence the concentration of this protein. Therefore, it is quite remarkable that tau decreases after storage for 12 days at 37 °C. At this temperature, the protein may be degraded by proteases, form aggregates, or undergo conformational changes, producing a form that is not detectable by one or both anti-tau antibodies used in the ELISA (10). Little is known about the relationship between CSF tau and temperature. The pathologic core protein of paired helical filaments, consisting of a portion of tau, is protease- and heat-resistant (19), but the aggregation of tau into paired helical filaments has been demonstrated to increase at temperatures above 30 °C (20). The tau protein is generally highly soluble, but the aggregated pathologic form found in neurofibrillary tangles in the brain might not be released in the CSF. The nature of tau in CSF is not well documented, but previous studies have revealed molecular masses of tau in lumbar CSF ranging from 25 to 80 kDa (21). The low-molecular-mass (25-kDa) form is not found in the brain, suggesting that tau is truncated when released into the CSF, probably as a result of degradation processes occurring in the brain. The truncated forms of tau in CSF should be well recognized by the anti-tau antibodies because they cover only a small part of the large full-length molecule. We speculate that storage of CSF for 12 days at 37 °C might lead to a change of the truncated form of tau into a more aggregated form, which is undetectable by the antibodies used in the ELISA (10).

In conclusion, both Aβ42 and tau are stable in CSF stored at −80 °C for a long period, but Aβ42 in CSF samples stored at 4, 18, and 37 °C decreased by ~20% during the first 2 days compared with the baseline value (−80 °C). Furthermore, the concentration of Aβ42 in CSF is influenced by the number of freeze/thaw cycles. To avoid these difficulties, it is best to process CSF as soon as possible after collection and store it at −80 °C for long storage. Preferably, CSF samples should be shipped on dry ice when stored frozen.

Niki S.M. Schoonenboom was partly funded through a grant from Alzheimer Nederland, Bunnik (V2001-023). Additional funding was received from the Stichting Alzheimer and Neuropsychiatry Foundation (Amsterdam, The Netherlands).

Appendix I: Estimation of Stability by the Arrhenius Method

The best way to determine the stability of analytes in body fluids is to perform a real-time stability experiment. This can be done by storing paired aliquots of a sample and measuring the concentrations of the proteins at certain time points, varying from some months to many years, taking into account that the sample is thawed once. However, this approach requires long experimental periods. Moreover, in real-time stability studies, protein measurements must be performed at the beginning, during, and at the end of the study. These measurements can often not be performed with the same batches of reagents. An increased interassay CV may be the result.

Therefore, estimation of protein stability should rather be performed using an accelerated stability testing protocol. The kinetics of protein denaturation are comparable to that of a first-order reaction, which means that the degradation rate is proportional to the concentration of the respective analyte. The equation for the accelerated stability testing protocol is:

$$-d[c]/dt = k[c]$$ or $$\ln c/c_0 = -kt$$

where $$c_0$$ is the initial protein concentration, $$c_t$$ is the concentration after time $$t$$, and $$k$$ is the rate constant. The rate constant, which is dependent on the temperature, is determined at three fixed temperatures, 4, 18, and 37 °C, assuming that at −80 °C the concentration remains constant during the time of the storage experiment. Afterward, the Arrhenius equation is applied, using the following formula:

$$\ln k_T = A + E/RT$$

in which $$A$$ is the preexponential factor and $$E/R$$ is the slope of the equation. Using the equation of the best-fit line, it is possible to calculate the degradation rate constant at each desired temperature:

$$\ln k_T = A + B/T$$

or

$$y = A + Bx$$
A and B have fixed values. By substituting the temperature of interest, e.g., −80 °C (193 K), \( k_{193} \) is calculated. We can calculate the time after which 90% or 95% of Aβ42 or tau can be recovered by substituting \( c_t/c_0 = 0.90 \) or 0.95 in the equation:

\[
\ln(0.90) = -k_{193}t \\
-0.105 = -k_{193}t \\
t = 0.105/k_{193}
\]

or

\[
\ln(0.95) = -k_{193}t \\
-0.0513 = -k_{193}t \\
t = 0.0513/k_{193}
\]

References


