FREE RADICAL RESEARCH, 2017
https://doi.org/10.1080/10715762.2017.1398403

Taylor & Francis
Taylor & Francis Group

ORIGINAL ARTICLE

Long-term stability of oxidative stress biomarkers in human serum

Eugène H. J. M. Jansen[a], Piet K. Beekhof[a], Dale Viezeliene[b], Vladimira Muzakova[c] and Jiri Skalicky[d]

[a]Centre for Health Protection, National Institute of Public Health and the Environment, Bilthoven, the Netherlands; [b]Department of Biochemistry, Lithuanian University of Health Sciences, Kaunas, Lithuania; [c]Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic; [d]Department of Clinical Biochemistry and Diagnostics, Regional Hospital of Pardubice, Pardubice, Czech Republic

ABSTRACT

The storage time and storage temperature might affect stability of oxidative stress biomarkers, therefore, they have to be analyzed after long-term storage of serum samples. The stability of three biomarkers reflecting oxidative stress: reactive oxygen metabolites (ROM) for hydroperoxides, total thiol levels (TTL) for the redox status and biological antioxidant potency (BAP) for the antioxidant status, was investigated at several time points during 60 months of storage at −20 and −80 °C. Biomarkers ROM and BAP showed a very good stability during storage for 60 months at both temperatures. In addition, the correlation of the data after 60 months of storage compared with the starting data was very good with correlation coefficients >0.9. The TTL assay showed good results in serum samples stored at −80 °C, but not in samples stored at −20 °C. Serum samples for analysis of the set of oxidative stress biomarkers ROM, BAP and TTL can be stored up to 60 months at −80 °C. ROM and BAP can also be stored at −20 °C during this period. The present results are very important for the biomarker-related epidemiological studies that make use of biobanks with samples stored for many years and for new project planning, including sample storage conditions.

ARTICLE HISTORY
Received 11 May 2017
Revised 17 October 2017
Accepted 25 October 2017

KEYWORDS
Antioxidant status; biomarkers; oxidative stress; redox status; storage stability

Introduction

Biomarkers reflecting oxidative stress are becoming increasingly important in epidemiological cancer studies and nutrition research. We developed a set of three biomarkers which can be used for large-scale epidemiological studies because the tests for their analysis have been programed into a clinical autoanalyzer. These biomarkers, which can be measured in serum and heparinised plasma, include reactive oxygen metabolites (ROM), total thiol levels (TTL) and biological antioxidant potency (BAP). The ROM assay detects lipid peroxidation products, such as hydro- and lipid peroxides [1]. The BAP assay measures total antioxidant status (TAS) of serum [2], and the TTL assay determines redox status by measuring free thiol groups in proteins [3].

In epidemiological studies, samples are often stored for several years at low temperatures. A prerequisite for a reliable analysis is the long-term stability of biomarkers in the samples. In the previous studies [4,5], we examined the stability of these biomarkers during 12 months of storage at −20 and −80 °C.

In the present study, the stability of the same set of oxidative stress biomarkers was tested in human serum samples stored for 60 months at two commonly used temperatures (−20 and −80 °C).

Material and methods

Human volunteers

For the stability study of ROM, TTL and BAP, serum samples of 34 human volunteers (blood donors) were used. The study was conducted with approval of the Ethics Committee of the Regional Hospital Pardubice (Czech Republic) under supervision of MU Dr Josef Hajek CSc, with written agreement of the volunteers. At the day of blood withdrawal the serum samples were prepared within 2 h and stored immediately at −20 °C. After 2 days, the frozen samples were shipped to the National Institute of Public Health and the Environment in Bilthoven on dry-ice, where the samples were divided into aliquots of 0.5 mL in 1.0 mL vials and stored at −20 and −80 °C until analysis. The freezers at −20 and −80 °C were equipped with an automatic temperature detection device with alarm function. During the whole study, no temperature problems were encountered.

CONTACT Eugène H. J. M. Jansen eugene.jansen@rivm.nl

Principles of the assays

The ROM assay for oxidative stress is a spectrophotometric test which determines the concentration of hydroperoxides in plasma. In the test, a plasma sample is diluted in an acidic solution to release iron ions from transferrin. The iron ions catalyze the breakdown of hydroperoxides which transforms a chromogenic substrate in coloured complex. The concentration of coloured complex is directly related to the hydroperoxide levels in the sample. In practice, a small amount of serum is diluted in an acidic solution (pH 4.8). In these conditions, iron ions become available to catalyze the breakdown of hydroperoxides to alkoxyl and peroxyl radicals. The chromogenic substrate (N,N-dimethyl-*p*-phenylenediamine) is transformed in a pink to red coloured radical cation. Quantitation is possible by means of a photometer (wavelength 505 or 546 nm). The concentration of coloured complex will be directly related to the hydroperoxides levels of the tested biological sample. In healthy subjects the d-ROMs has a value between 250 and 300 UNIT CARR (U CARR), which also represents the normal range. Values above 300 U CARR are indicative of a condition of oxidative stress.

The BAP assay is a spectrophotometric test which determines the concentration of antioxidants in plasma. The BAP assay is based on the ability of a coloured solution, containing ferric (Fe^{3+}) ions adequately bound to a special chromogenic substrate (ammonium thiocyanate), to decolour when Fe^{3+} ions are reduced to ferrous (Fe^{2+}) ions as well as it can be observed by adding a reducing system, i.e. a serum sample. In healthy subjects, the BAP assay assumes a value higher than 2200 µmol/L is considered as the optimum value. Values less than 2200 µmoles/L are indicative of a condition of oxidative stress by lowering of antioxidant defenses.

The TTL assay is a spectrophotometric test which determines the concentration of free thiol groups in plasma. The test is based on the ability of free thiol groups to develop a coloured complex (absorbance at 405 nm) when reacted with 5,5-dithiobis-2-nitrobenzoic acid. The colour intensity is directly related to the thiol groups in the sample which are not affected by oxidation. L-cysteine has been used as standard.

Measurements of biomarkers

The biomarkers ROM and TTL, were determined in serum with autoanalyzers: the clinical autoanalyzer LX20 Pro was used for the measurements after 1 and 2 years of sample storage and another clinical autoanalyzer Dx 800 for the measurements after 2 years (both from Beckman-Coulter, Woerden, the Netherlands). ROM and BAP assays were obtained from Diacron, Grosseto, Italy and TTL assay was obtained from RelAssay, Gaziantep, Turkey. All assays were adapted for autoanalyzers. The average interassay coefficients of variation were 5.9% for ROM, 3.8% for TTL and 2.8% for BAP.

The initial concentrations of the parameters at Day 0 (prior to storage) were determined twice on the day of storage. At the other time points, measurements were performed as a single measurement. At each time point, a fresh sample was taken from the −20 and −80 °C freezers and used for analysis. In the measurements at each time point, fresh standards and quality control (QC) samples (dROMs Calibrator, Diacron) were used for all three assays, and the concentration of the biomarkers in the samples were corrected if appropriate.

Statistics

In the figures, the concentrations at each time point were expressed as a percentage of the mean value at day 0. Statistical analysis with repeated measure one-way analysis of variance (ANOVA) using GraphPad Prism was performed to determine whether the concentrations at certain time points were statistically different from that at Day 0.

Results

ROM assay

The stability of ROM was very good during 60 months of storage at −20 and −80 °C (Figure 1). Although some minor changes were observed at some time points, there were no statistically significant differences between the data of both temperatures at all-time

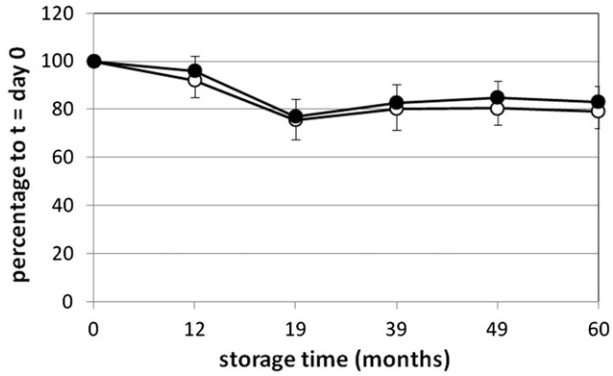

Figure 1. Stability of ROM assay performed in human serum samples after storage at −20 °C (o) and −80 °C (•). Shown are the mean values − the standard variations for −20 °C and + the standard variations for −80 °C, expressed as percentages to the individual values of the 34 volunteers as determined before the storage at low temperatures.

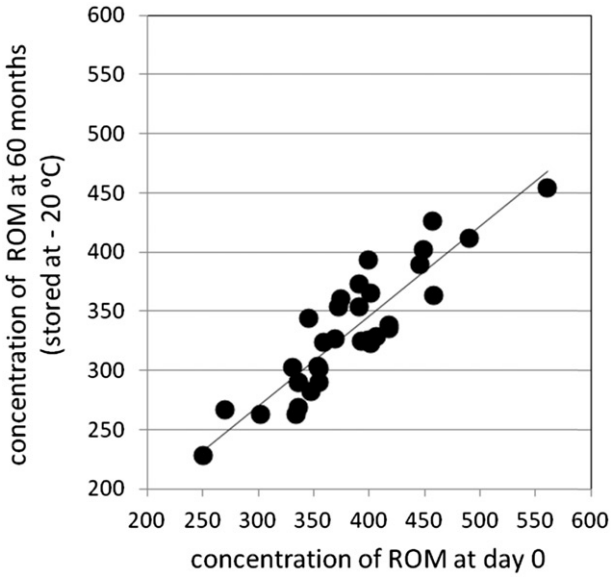

Figure 2. Correlation between the ROM concentrations (expressed in IU/L) in serum samples of 34 volunteers, measured at Day 0 and in serum samples stored for 60 months at −20 °C. The correlation coefficient (R^2) of the regression line was 0.806 with a p value of a t-test between the two sample sets of 7.68 e^{-4}.

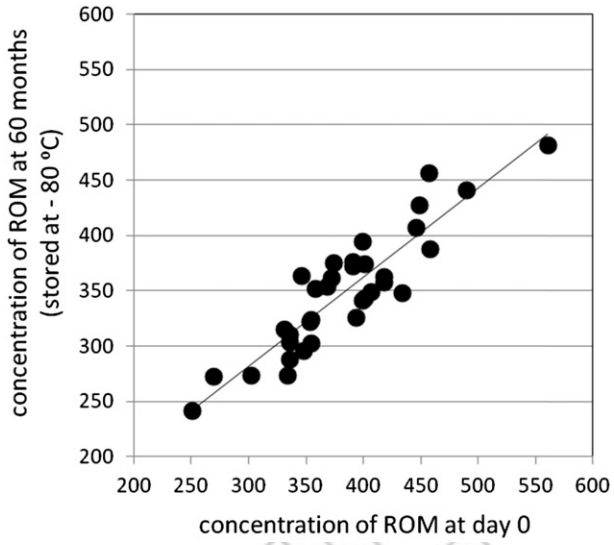

Figure 3. Correlation between the ROM concentrations (expressed in IU/L) in serum samples of 34 volunteers, measured at Day 0 and in samples stored for 60 months at −80 °C. The correlation coefficient (R^2) of the regression line was 0.820 with a p value of a t-test between the two sample sets of 0.017.

points. Data of intermediate time points during the first 12 months of storage was reported earlier [4]. It must be noted that from the Month 19 and onwards, the company Diacron has made a change in the assay procedure, and that probably caused a shift to somewhat lower concentrations of ROM.

The good stability of the ROM assay was proved by correlation of the ROM concentrations in the individual samples at Day 0 with those stored for 60 months. At −20 °C (Figure 2), the equation of the linear regression line is $y = 0.76x + 42$ with a good correlation coefficient (R) of 0.898. The correlation between the ROM concentrations in serum samples measured at $t = 0$ and in samples stored for 60 months at −80 °C is shown in Figure 3. Here, a good correlation was also found with the equation of the linear regression line $y = 0.81x + 40$, with a correlation coefficient R of 0.906.

Also, the correlation between the ROM concentrations in serum samples measured after 60-month storage at −20 and −80 °C was very good.

The equation of the linear regression line was $y = 1.05x - 0.37$, with a correlation coefficient R of 0.990 (Figure 4).

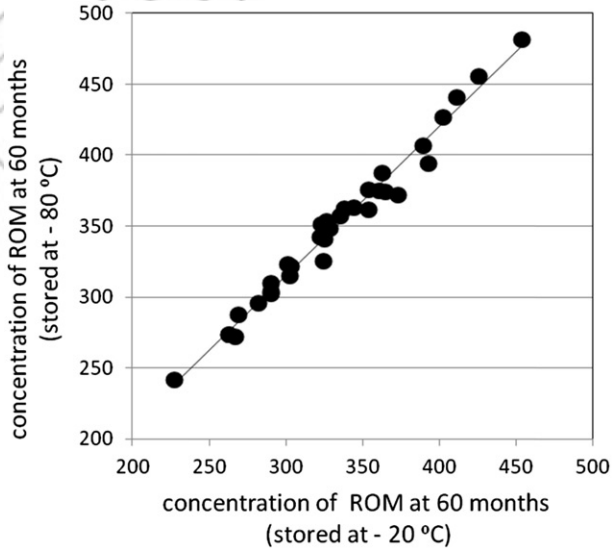

Figure 4. Correlation between the ROM concentrations (expressed in IU/L) in serum samples of 34 volunteers, measured after 60-month storage at −20 and −80 °C. The correlation coefficient (R^2) of the regression line was 0.980 with a p value of a t-test between the two sample sets of 0.215.

Total thiol assay

The storage stability of TTL was not very good during 60-month storage at −20 °C. After 1 month, there already was a decrease in the TTL concentration in the samples stored at −20 °C compared with the samples stored at −80 °C (Figure 5). The data at intermediate time points until 12 months were already presented in the previous study [4], and the difference of TTL concentrations in samples stored at both temperatures became even more pronounced during the length of storage. The levels of TTL in samples stored at −80 °C increased in time. It cannot be excluded that this

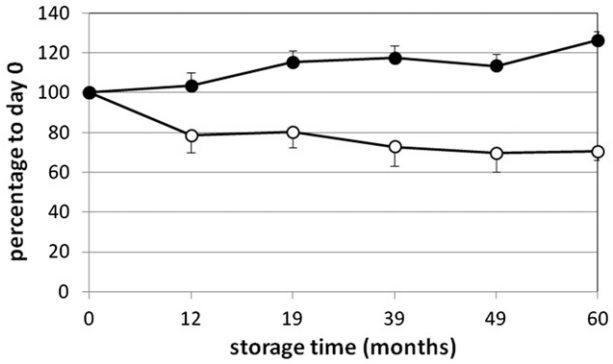

Figure 5. Stability of the TTL assay performed in human serum samples after storage at −20 °C (o) and −80 °C (•). Shown are the mean values – the standard variations for −20 °C and + the standard variations for −80 °C, expressed as percentages to the individual values of the 34 volunteers as determined before the storage at low temperatures.

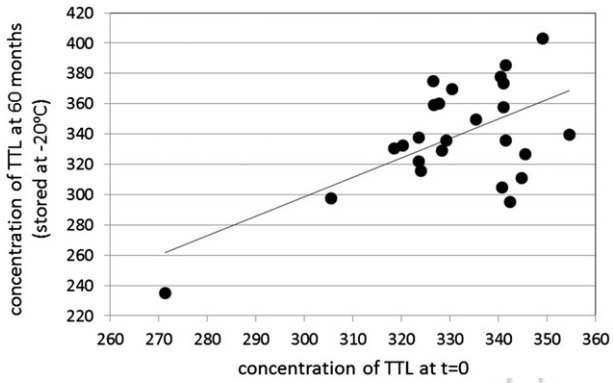

Figure 6. Correlation between the TTL concentrations (expressed in µmol/L) in serum samples of 34 volunteers, measured at Day 0 and in samples stored for 60 months at −20 °C. The correlation coefficient (R^2) of the regression line was 0.366 with a p value of a t-test between the two sample sets of 0.357.

increase is real, although it is also possible that the calibration standard (a cysteine solution) supplied with the kits caused this increase.

To check whether the lower stability at −20 °C also influenced the TTL concentration rank order of the samples, a correlation was made between the concentrations at both temperatures. The correlation of TTL concentrations in individual samples at Day 0 with those stored for 60 months at −20 °C is shown in Figure 6. From this figure, it can be seen that the correlation is not very good with the equation of the linear regression line $y = 1.28x - 85$ and a correlation coefficient R of 0.605.

In Figure 7, the correlation between the TTL concentrations in serum samples measured at $t = 0$ and in samples stored for 60 months at −80 °C is shown. Here, a

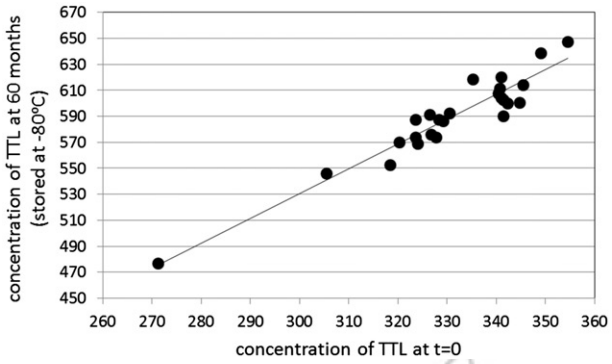

Figure 7. Correlation between the TTL concentrations (expressed in µmol/L) in serum samples of 34 volunteers, measured at Day 0 and in samples stored for 60 months at −80 °C. The correlation coefficient (R^2) of the regression line was 0.911 with a p value of a t-test between the two sample sets of 1.53 e^{-35}.

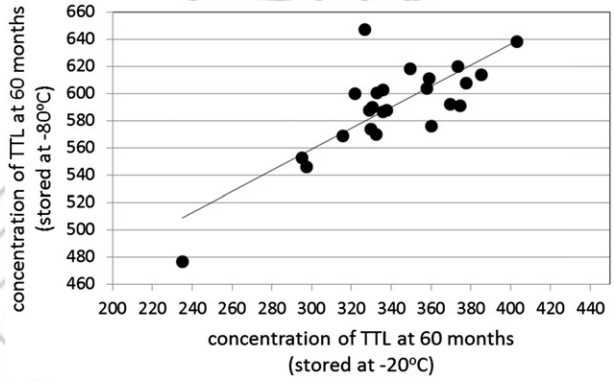

Figure 8. Correlation between TTL concentrations (expressed in µmol/L) in serum samples of 34 volunteers, measured after 60-month storage at −20 and −80 °C. The correlation coefficient (R^2) of the regression line was 0.630 with a p-value of a t-test between the two sample sets of 1.88 e^{-28}.

much better correlation was found with the equation of the linear regression line $y = 1.91x - 42$, with a correlation coefficient R of 0.955.

However, the correlation between the TTL concentrations in serum samples measured after 60-month storage at −20 and −80 °C was not very good. The equation of the linear regression line was $y = 0.77x + 328$ with a correlation coefficient R of 0.795 (Figure 8).

BAP assay

The stability of BAP was very good during 60 months at the temperatures −20 and −80 °C (Figure 9). Data of the stability during the first 12 months have been reported earlier [5]. Although some minor changes were observed at some time points, there are no statistically

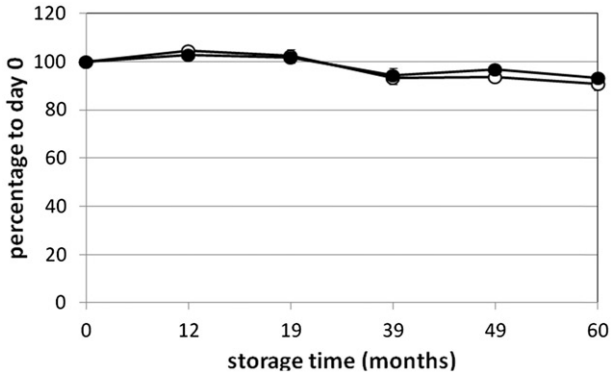

Figure 9. Stability of the BAP assay performed in human serum samples during storage at −20 °C (o) and −80 °C (•). Shown are the mean values – the standard variations for −20 °C and + the standard variations for −80 °C, expressed as percentages to the individual values of the 34 volunteers as determined before the storage at low temperatures.

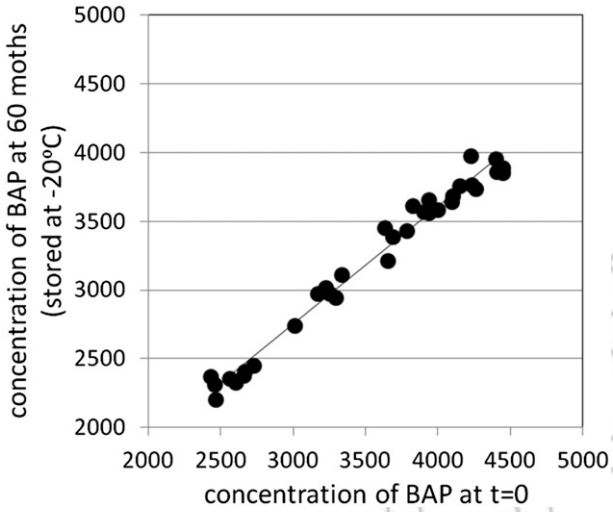

Figure 10. Correlation between the BAP concentrations (expressed in IU/L) in serum samples of 34 volunteers, measured at $t = 0$ and in samples after 60-month storage at −20 °C. The correlation coefficient (R^2) of the regression line was 0.980 with a p value of a t-test between the two sample sets of 0.041.

significant differences between the data of two temperatures.

The stability of the BAP was checked in another way using correlation of the concentrations in individual samples at Day 0 with those stored for 60 months at −20 °C (Figure 10). The equation of the linear regression line is $y = 0.86x + 183$ with a good correlation coefficient R of 0.990.

In Figure 11, the correlation between the BAP concentrations in serum samples measured at Day 0 and in samples stored for 60 months at −80 °C is shown. Here, a good correlation was also found with the equation of the linear regression line $y = 0.82x + 391$; a correlation coefficient R of 0.992.

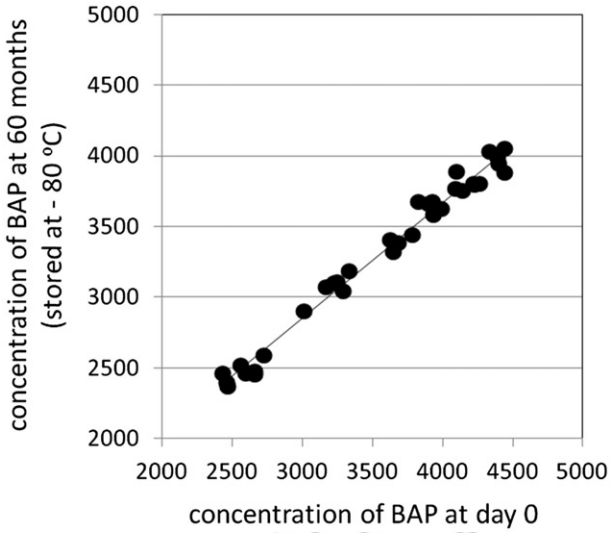

Figure 11. Correlation between the BAP concentrations (expressed in IU/L) in serum samples of 34 volunteers, measured at Day 0 and in samples after 60-month storage at −80 °C. The correlation coefficient (R^2) of the regression line was 0.983 with a p value of a t-test between the two sample sets of 0.136.

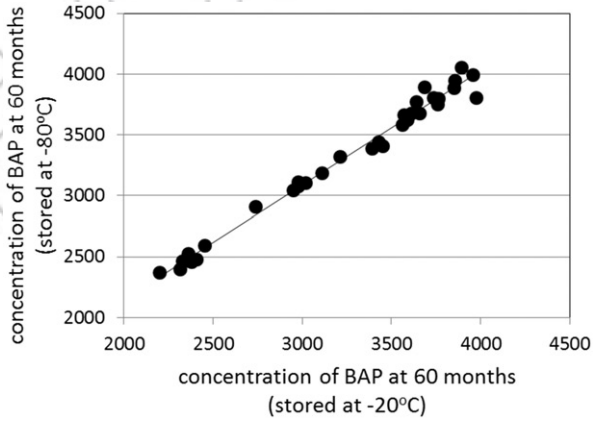

Figure 12. Correlation between the TTL concentrations (expressed in IU/L) in serum samples of 34 volunteers, measured after 60-month storage at −20 and −80 °C. The correlation coefficient (R^2) of the regression line was 0.986 with a p value of a t-test between the two sample sets of 0.603.

Also, the correlation between the BAP concentrations in serum samples measured after 60-month storage at −20 and −80 °C was very good.

The equation of the linear regression line was $y = 0.94x + 258$; a correlation coefficient R of 0.993 (Figure 12).

The correlation coefficients between the different conditions of storage are summarized in Table 1. As was stated before, the ROM and BAP tests showed a good stability at both temperatures, whereas the stability of TTL was much less at −20 °C, which was reflected by the lower correlation coefficients with samples stored at −20 °C.

Table 1. Correlation coefficients between the various series of serum samples stored for 60 months.

Biomarker	Day 0 versus Month 60 (stored at −20 °C)	Day 0 versus Month 60 (stored at −80 °C)	Temperature −20 versus −80 °C (stored for 60 months)
ROM	0.898	0.906	0.990
BAP	0.990	0.992	0.993
TTL	0.610	0.958	0.795

Discussion

Some studies on oxidative stress used biomarkers of oxidatively generated damage as intermediate outcomes. Excessive reactive oxygen species, produced as a result of an unhealthy lifestyle, cause oxidatively generated damage of various macromolecules, such as DNA, lipids and proteins, contributing to the onset of several chronic diseases [6].

Detection of intermediate reactive oxygen species, such as intermediate lipid radicals or antioxidant radicals (vitamin E radical), is not an easy task mainly because they are reactive compounds, with a very short lifespan in the order of nano- or microseconds. Some exceptions are hydrogen peroxide and organic hydroperoxides. These compounds can have a longevity of minutes and, therefore, can travel across membranes into the circulation or other tissues.

ROM assay

The ROM assay is based on iron-based oxidation reaction in which the existing hydro- or lipid peroxides are detected colorimetrically (Figure 13). ROM appears as a biomarker of disease in cancer research [7–10], cardiovascular studies [11–13] and in aging studies [14–16]. The assessment of the ROM assay reliability was performed earlier with respect to the short-term handling in daily use [17] and after storage for up to 1 year [4].

Present study showed that the ROM assay was very reliable in serum samples that were stored for 60 months. Storage at −20 °C gave somewhat less accurate results in correlation with the initial concentrations than samples stored at −80 °C. However, it can be concluded that storage at −20 °C is also suitable for this assay.

There is only one other reported study investigating ROM in serum but only during 3-month storage at −20 °C [18].

TTL assay

The biomarker TTL for the redox status in serum determines mainly free thiol groups in proteins [19,20]. Under oxidative stress conditions, the oxidized

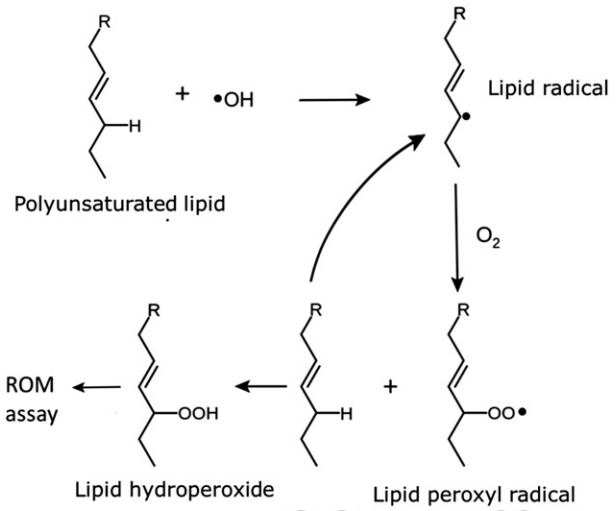

Figure 13. Schematic representation of the formation of stable lipid hydroperoxides by oxidative stress processes from lipid peroxidation, that can be detected by the ROM assay in serum samples.

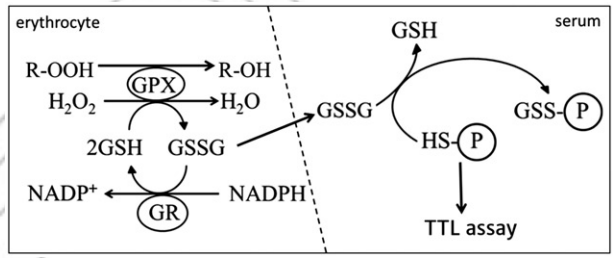

Figure 14. Schematic representation of thiol metabolism in erythrocytes producing hydrogen peroxides which oxidizes glutathione, which is reduced in serum on account of oxidation of protein-bound thiols which are detected with the TTL assay. GPX: glutathione peroxidase, GR: glutathione reductase, NADP: nicotinamide adenine dinucleotide phosphate, GSH: glutathione, GSSG: oxidized glutathione, P-SH: thiol groups of proteins, P-SSG: oxidized thiol groups of proteins.

glutathione reacts with free sulfhydryl groups of the cysteine side chains in proteins. This is used for the TTL assay [21], which detects the decreased amount of SH-groups indicating lowered redox capacity (Figure 14), as was shown in the large-scale aging studies [14,15].

The present study showed that serum samples must be stored at lower temperatures for TTL assay than for the other two, ROM and BAP, assays. Therefore, for determination of redox status, it is recommended to store serum samples at −80 °C instead of −20 °C.

BAP assay

The assay of BAP, as a biomarker for the antioxidant status, was chosen because of the relatively low contribution of uric acid to the TAS compared with other total

antioxidant assays [2], such as the TAS assay of different performances [22–26] and the FRAP assay [27].

This assay showed a very good stability of BAP during 60 months of study. Also, less problems were encountered in the daily performance of the assay and QC parameters including the coefficient of variation as compared with the other two assays ROM and TTL.

In conclusion, serum samples can be stored for longer periods up to 5 years without any effect on the reliability of the BAP assay.

General conclusions

From the present study, it is clear that the ROM and BAP assays are very reliable for serum samples that were stored at both −20 and −80 °C, although storage at −80 °C has some advantages in precision of the ROM assay.

The biomarker for the redox status assay (TTL) is very stable in serum samples stored at −80 °C, but less so in samples stored at −20 °C.

In this study, the serum samples have been stored for 60 months, but there are no indications that storage for even longer periods would be unacceptable.

In general, serum or plasma should be preferably stored for longer periods at low temperatures (−70 to −80 °C). Storage should also be done of samples in separate aliquots which can be used for analyses at different time points or a different set of biomarkers. In addition, much attention should also be given to the QC if the biomarkers are determined at different time points.

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgements

Part of the study was conducted with the financial support from the board of Directors of the National Institute of Public Health and the Environment (Bilthoven, the Netherlands), within the Strategic project on Antioxidant supplements for aging.

References

1. Carratelli M, Porcaro L, Ruscica M, De Simone E, Bertelli AA, Corsi MM. Reactive oxygen Metabolites and prooxidant status in children with Down's syndrome. Int J Clin Pharmacol Res 2001;21:79–84.
2. Jansen EH, Ruskovska T. Comparative analysis of serum (anti)oxidative status parameters in healthy persons. Int J Mol Sci 2013;14:6106–6115.
3. Karadag-Oncel E, Erel O, Ozsurekci Y, Caglayik DJ, Kaya A, Gozel M, et al. Plasma oxidative stress and total thiol levels in Crimean-Congo hemorrhagic fever. Jpn J Infect Dis 2014;67:22–26.
4. Jansen E, Beekhof P, Viezeliene D, Muzakova V, Skalicky J. Long-term stability of cancer biomarkers in human serum: biomarkers of oxidative stress and redox status, homocysteine, CRP and the enzymes ALT and GGT. Biomark Med 2015;9:425–432.
5. Jansen EHJM, Beekhof PK, Cremers JWJM, Viezeliene D, Muzakova V, Skalicky J. Long-term stability of parameters of antioxidant status in human serum. Free Radic Res 2013;47:535–540.
6. Evans MD, Dizdaroglu M, Cooke MS. Oxidative DNA damage and disease: induction, repair and significance. Mutat Res 2004;567:1–61.
7. Leufkens AM, van Duijnhoven FJB, Woudt SHS, Siersema PD, Jenab M, Jansen EH, et al. Biomarkers of oxidative stress and risk of developing colorectal cancer: a cohort-nested case-control study in the European Prospective Investigation Into Cancer and Nutrition. Am J Epidemiol 2012;175:653–663.
8. Aleksandrova K, Drogan D, Boeing H, Jenab M, Bueno-de-Mesquita HB, Jansen E, et al. Adiposity, mediating biomarkers and risk of colon cancer in the European Prospective Investigation into Cancer and Nutrition Study. Int J Cancer 2014;134:612–621.
9. Aleksandrova K, Jenab M, Bueno-de-Mesquita HB, Fedirko V, Kaaks R, Lukanova A, et al. Biomarker patterns of inflammatory and metabolic pathways are associated with risk of colorectal cancer: results from the European Prospective Investigation into Cancer and Nutrition (EPIC). Eur J Epidemiol 2014;29:261–275.
10. Schöttker B, Zhang Y, Heiss JA, Butterbach K, Jansen EHJM, Bewerunge-Hudler M, et al. Discovery of a novel epigenetic cancer marker related to the oxidative status of human blood. Genes Chromosomes Cancer 2015;54:583–594.
11. Schöttker B, Saum KU, Jansen EHJM, Holleczek B, Brenner H. Associations of metabolic, inflammatory and oxidative stress markers with total morbidity and multimorbidity in a large cohort of older German adults. Age Ageing 2016;45:127–135.
12. Vassalle C, Bianchi S, Battaglia D, Landi P, Bianchi F, Carpeggiani C. Elevated levels of oxidative stress as a prognostic predictor of major adverse cardiovascular events in patients with coronary artery disease. J Atheroscler Thromb 2012;19:712–717.
13. Vassalle C, Pratali L, Boni C, Mercuri A, Ndreu R. An oxidative stress score as a combined measure of the pro-oxidant and anti-oxidant counterparts in patients with coronary artery disease. Clin Biochem 2008;41:1162–1167.
14. Schöttker B, Brenner H, Jansen EHJM, Gardiner J, Peasey A, Kubínová R, et al. Evidence for the Free radical/Oxidative Stress Theory of ageing from the CHANCES consortium: a meta-analysis of individual participant data. BMC Med 2015;13:300.
15. Saum KU, Dieffenbach AK, Jansen EHJM, Schöttker B, Holleczek B, Hauer K, Brenner H. Association between oxidative stress and frailty in an elderly German

population: results from the Esther cohort study. Gerontology 2015;61:407–415.
16. Schöttker B, Saum KU, Jansen EHJM, Boffetta P, Trichopoulou A, Holleczek B, et al. Oxidative stress markers and all-cause mortality at older age: a population-based cohort study. J Gerontol A Biol Sci Med Sci 2015;70:518–524.
17. Jansen EHJM, Beekhof PK, Cremers JWJM, Viezeliene D, Muzakova V, Skalicky J. Short-term stability of biomarkers of oxidative stress and antioxidant status in human serum. ISRN Biomarkers 2013; 2013:316528.
18. Iamele L, Fiocchi R, Vernocchi A. Evaluation of an automated spectrophotometric assay for reactive oxygen metabolites in serum. Clin Chem Lab Med 2002;40:673–676.
19. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70–77.
20. Hu ML. Measurement of protein thiol groups and glutathione in plasma. Meth Enzymol 1994;233: 380–385.
21. Costa CMd, Santos RCCd, Lima ES. A simple automated procedure for thiol measurement in human serum samples. J Bras Patol Med Lab 2006;42:345–350.
22. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. Method for the measurement of antioxidant activity in human fluids. J Clin Pathol 2001;54:356–361.
23. Young IS. Measurement of total antioxidant capacity. J Clin Pathol 2001;54:339.
24. Cao G, Prior RL. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. Clin Chem 1998;44:1309–1315.
25. Lamont J, Campbell J, FitzGerald P. Measurement of individual vs total antioxidants. Clin Chem 1997;43:852–854.
26. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin Biochem 2004;37:277–285.
27. Benzie IFF, Strain JJ. The ferric Reducing Ability of plasma (FRAP) as a measure of "Antioxidant Power": the FRAP Assay. Anal Biochem 1996;239:70–76.