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Calorimetric and spectroscopic studies characterization of newborn rat' blood serum after maternal administration of cyclophosphamide

Zofia Drzazga · Katarzyna Michalik · Tomasz Halat · Anna Michnik · Henryk I. Trzeciak

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Abstract Differential scanning microcalorimetry (DSC) and UV–VIS absorption spectroscopy were used to obtain the characteristics of blood serum from newborn rat' after maternal treatment with cyclophosphamide in comparison with control. The obtained DSC curves reveal a complex endothermic peak due to the unfolding process of various serum proteins. Thermal profiles and absorption spectra of blood serum are sensitive to the age of newborns as well as to effect of maternal administration of cyclophosphamide. The most significant disturbances in serum proteome were observed for 14-day old newborns. The thermodynamic parameters: enthalpy change (ΔH), the normalized first moment (M_1) of the thermal transition with respect to the temperature axis and the ratio of C_p^{ex} at 70 and 60 °C describing denaturation contributions of globulin forms in respect to unliganded albumin with haptoglobin was estimated. Moreover, the second derivative spectroscopy in the UV region was used to resolve the complex protein spectrum. The differences in blood serum detected by DSC and UV–VIS confirm a potential usefulness of these methods for diagnostic and monitoring changes with age as well as the pathological state of blood serum.

Z. Drzazga · K. Michalik (✉) · T. Halat · A. Michnik
Chełkowski's Institute of Physics, Department of Medical Physics, University of Silesia, ul. Uniwersytecka 4, 40007 Katowice, Poland
e-mail: kasia_michalik1@wp.pl

H. I. Trzeciak
Department of Pharmacology, Faculty of Pharmacy, Medical University of Silesia, ul. Jagiellońska 4, 41200 Sosnowiec, Poland

H. I. Trzeciak
Cardinal August Hlond Upper Silesian College of Pedagogical Education, ul. Powstańców 19, 41400 Mysłowice, Poland

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Introduction

Calorimetric techniques giving unique thermodynamic signature for individual proteins and binding interactions have become a useful method in the research of analytical medical problems such as degeneration of human cartilage [1] and muscle [2]. Special focus has been given to changes in blood serum [3–5].

It is thought that blood serum proteins make up 6–8% of the blood and are about equally divided between serum albumins and the great variety of serum globulins. Changes in concentration of blood serum proteins, their structure and binding properties can lead to pathological states such as cancer [6] and Alzheimer disease [7].

Cancer is one of the most common causes of death during the reproductive years [8]. When cancer occurs in pregnancy there is almost always a conflict between optimal maternal therapy and foetal well-being. Consequently, either maternal or foetal health or both may be endangered. If chemotherapeutic agents cross the placenta the foetus is exposed and this may lead to mutagenic effects especially to somatic cells, causing gene mutations and chromosomal breaks [9, 10]. Knowledge about the side effects resulting from the use of antineoplastic drugs during pregnancy is limited. Cyclophosphamide (CP) is an anti-cancer chemotherapy drug that is used primarily for treating several types of cancer, e.g., lymphoma, leukaemia, retinoblastoma, and cancers of the breast and ovary [11, 12]. This medication is classified as an "alkylating agent" and as D in the FDA pregnancy category, e.g., cyclophosphamide

can be harmful to an unborn baby. CP may disturb normal growth and development by affecting protein degradation. Alkylation of neonatal proteins for example may alter the conformation of proteins thus rendering these molecules more susceptible to degradation [13].

This article describes the DSC and UV–VIS studies in vitro of newborn rat's blood serum during the first month of life after maternal administration of CP.

Experimental

Materials

The experiments were carried out on blood serum obtained from Wistar newborn rats (6–7 rats in each group) whose mothers were fed a standard diet ad libitum. The rats were delivered by the Centre of Experimental Medicine of the Medical University of Silesia. The procedure of the experiments on animals was approved by the Local Ethics Commission in Katowice, Poland. Pregnant rats were obtained by caging a proestrus/oestrus female overnight with a male of proven fertility. Females showing a vaginal plug of the presence of spermatozoa in the vaginal smear were designated pregnant and housed in separate cages. The rats were divided into two groups. The first group of rats (CP group) received cyclophosphamide (Asta Medica) at a dose of 5 mg/kg ip. on 5, 10, and 15th day of pregnancy. The second group: the control female rats received 0.9% NaCl solution or water at the same volume of 2 or 1 mL/kg, respectively. The blood serum was obtained from 7-, 14- and 28-day old newborn rats. The samples were diluted 10-fold in degassed buffer KH_2PO_4/Na_2HPO_4 (Sigma–Aldrich) to the final pH equal 7.2. Protein concentration was determined according to its amount in dry mass of the serum sample.

Methods

Differential scanning calorimetry (DSC) scans were performed using the VP DSC ultrasensitive microcalorimeter (MicroCal Inc., Northampton, MA) with cell volumes of 0.5 mL. Heat capacity versus temperature profiles were obtained for scanning rate of 1.0 K min^{-1} in temperature range 25–100 °C. Additionally constant pressure of about 1.8 atm was exerted on the liquids in the cells. The calorimetric data were corrected for the instrumental baseline buffer–buffer. Samples were normalized for the gram mass of protein. A linear baseline was used to obtain the excess apparent molar heat capacity C_p^{ex} (J $°C^{-1}$ g^{-1}).

The DSC measurements were supported by optical spectroscopy. UV–VIS absorption spectra were obtained with the use of HR4000 spectrometer (Ocean Optics HR4C 1970).

Analysis

DSC curves were analyzed with the use of MicroCal Origin and the UV–VIS spectra by means SpectraSiute software. Statistical analysis of the results was done with Statistica 7.1 using ANOVA. Schapiro-Wilk and Levene tests were performed to check the normality of the distributions and homogeneity of the variance, respectively. Differences with $p < 0.05$ were regarded as significant.

Results and discussion

Overall characteristics of the control group of newborns in relation to their age

Figures 1 and 2 present UV–VIS spectra and DSC curves for solution of blood serum from 7- and 28-day old newborn rats, respectively. The averaged absorption spectra (Fig. 1) show the broad band with maximum at about 278 nm and local minima at about 252 nm associated with the presence of aromatic amino acids such as tryptophan (Trp), tyrosine (Tyr), and phenyloanaline (Phe) originating from protein fractions—mainly albumins and globulins. The additional peak appearing at about 410 nm (Soret band) can be connected with the remaining haemoglobin forms [14]. Moreover, one can see that absorption spectra become more distinct with the age of newborns.

The representative DSC curves of blood serum obtained from 7- and 28-day old newborns (Fig. 2) reveal complex endothermic peak due to the unfolding process of various serum proteins. It was reported that mammalian blood proteins exhibit similarities in TG/DTG and DSC profiles

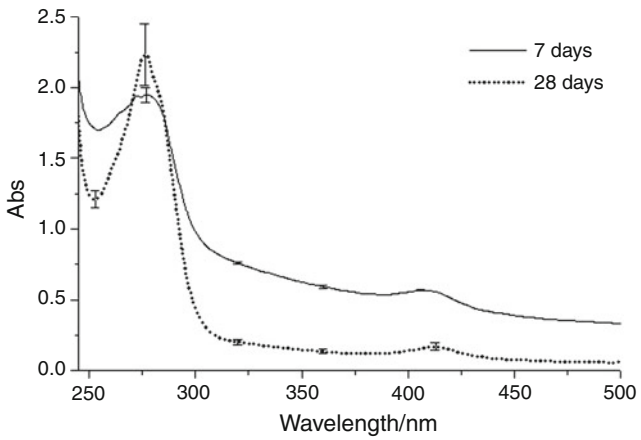

Fig. 1 Averaged absorption spectra of control blood serum for 7- and 28-day old newborn rats

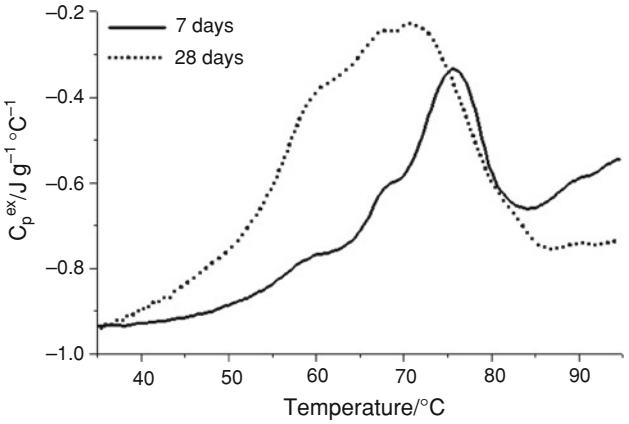

Fig. 2 The representative raw DSC curves of control blood serum from of 7- and 28-day old newborn rats

although they can differ in their unfolding pathway [15–17]. It was shown by Garbet et al. [4] that DSC curves of blood plasma represent the sum of weighted contributions of an individual protein. The major input to the measured heat effect is ascribed to albumins, gamma globulins including immunoglobulins such as IgG and IgM, α globulins with haptoglobin, $\alpha 2$ macroglobulin, and β globulins with transferrin. Taking into consideration similarities of the plasma/serum proteome of human and rat one can expect an analogous denaturation process of abundant serum proteins. It has been shown that human and rat albumin exhibit close endotherms [16] suggesting similar mechanism of unfolding. Therefore, in the obtained DSC curves (Fig. 2) albumins and haptoglobins seem to contribute to the local maximum of C_p^{ex} in the temperatures at about 60 °C. However, the main maximum of DSC runs occurring in temperature above 65 °C can be mainly affected by $\alpha\beta$ and γ globulin fractions [18]. Additionally the denaturation process in this range can be slightly influenced by the haemoglobin forms [19] due to the contamination of the rat's blood serum as can be seen in UV–VIS spectra (Fig. 1). Comparison of DSC results for human blood serum/plasma presented in Garbett et al. [3] and Michnick et al. [5] with rat blood serum obtained by us yields a different signature of their DSC runs. To better insight into problem the ratio of C_p^{ex} at 70 and 60 °C describing denaturation contributions of globulin forms in respect to unliganded albumin with haptoglobin (analogously as in work [5]) was calculated. It follows from Table 1 that the excess heat capacity arising from proteins subject to denaturation at higher temperatures is more distinct for newborn rat serum ($C_p^{ex}(70)/C_p^{ex}(60) > 1$) than for human serum ($C_p^{ex}(70)/C_p^{ex}(60) < 1$) [5].

It should be noted that blood serum characteristics are influenced by the age of the newborns. There are large differences in DSC thermal transition profiles of blood serum from 7- and 28-days old newborns (Fig. 2). The change of the shape is accompanied by a marked shift of the main maximum towards lower temperatures with the age of the newborns (see also data in Table 1).The two weakly shaped local maxima at about 60 and 67 °C on the low temperature shoulder are also modified. The analysis of ratio C_p^{ex70}/C_p^{ex60} listed in Table 1 suggests that the albumin contributions are markedly bigger for 28-day newborns than for 7-day ones. This is in agreement with immunoelectrophoresis measurements which point to the increase of albumin and transferrin content with rat age [20]. The changing content of other constituents, e.g., various forms of globulins with rat age [21] cannot be neglected in factor C_p^{ex70}/C_p^{ex60} either.

Additionally, the normalized first moment (M_1) of the thermal transition with respect to the temperature axis were calculated according to Eq. 1:

$$M_1 = \frac{\int_{T_1}^{T_2} TC_p dT}{\int_{T_1}^{T_2} C_p dT} \quad (1)$$

The decreasing values of M_1 with rat' age (Table 1) confirm the increasing role of proteins with unfolding events occurring in lower temperatures.

Figure 3 displays an increase of the basic thermodynamic parameter: the specific enthalpy change (ΔH) of serum denaturation (calculated as the area under DSC curve) with age. The differences between 14- and 28-day newborns are statistically essential.

Table 1 The comparison of thermodynamic parameters (temperature of peak maximum T_m, calculated moments M_1/°C and $C_p^{ex\ 70}/C_p^{ex\ 60}$ (mean ± standard deviation)) of blood serum denaturation process as a function of rat's age and maternal cyclophosphamide (CP) administration

Days	T_m/°C		M_1/°C		$C_p^{ex\ 70}/C_p^{ex\ 60}$	
	Control	CP	Control	CP	Control	CP
7	75.2 ± 0.3	75.1 ± 0.2	70.4 ± 0.1	69.9 ± 0.2	2.4 ± 0.2	1.8 ± 0.1
14	70.7 ± 0.2	76.0 ± 0.4	69.3 ± 0.3	73.2 ± 0.2	3.0 ± 0.1	9.0 ± 0.1
28	70.5 ± 0.2	67.5 ± 0.1	65.9 ± 0.9	67.1 ± 0.1	1.3 ± 0.2	1.6 ± 0.1

Cyclophosphamide effect

Figure 4a–c, respectively, present thermal characteristics of blood serum obtained from 7-, 14- and 28-day old newborn rats whose mothers were treated with cyclophosphamide versus the control group. From the above it follows that maternal administration of CP causes changes in DSC profiles for all age groups. For better insight into the problem differential curves between CP and control group are show as an insert. The difference in the denaturation process can be analyzed in two temperature ranges 40–65 °C and above 65 °C. In the first region the minor differences in amplitude of DSC curves for 7-day rats becomes more visible with the age of the newborns. In the temperature range 65–85 °C a marked increase of C_p^{ex} is observed for 7- as well as 28-day old ones (Fig. 4a, c).

Special attention should be paid to the decomposition process of serum blood from 14-day newborn rats. An unexpected strong C_p^{ex} increase forming a double peak (at about 67 and 76 °C) (seen also in the differential curves) should be noted. There is an increase of amplitude of the peaks occurring at temperature above 65 °C with a concomitant decrease in amplitude of the local peak at about 60 °C. The ratio C_p^{ex70}/C_p^{ex60} indicates a large disturbance in the equilibrium of thermal denaturation of the individual proteins within serum.

Visible anomalies in DSC profiles of serum denaturation (Fig. 4b) as well as the distinct changes in UV–VIS spectra (Fig. 5) noted for 14-day rats seems to be partly explained by postnatal changes in the newborns' organism. Breast-feeding of newborns is terminated 12 days after their birth. The transition from maternal milk to dry food results in a period of starvation. It was reported by Dobrowolski et al. [22] that low nutrient intake during the first days after weaning is the main cause of sudden changes in the serum blood plasma composition and the interactions within. Maternal treatment with cyclophosphamide may enhance this effect. The significant changes in the DSC curves of plasma from diseased individuals were suggested in [3] to result rather from interactions of small molecules or

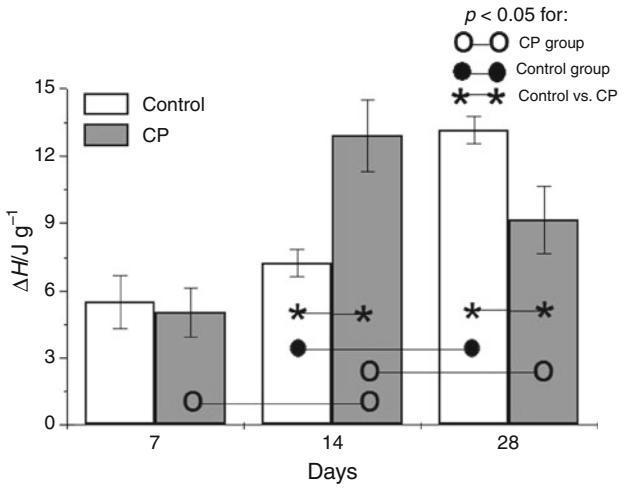

Fig. 3 Enthalpy change ΔH (mean and standard deviation) of blood serum for control and cyclophosphamide (CP) group as a function of a newborn rat's age

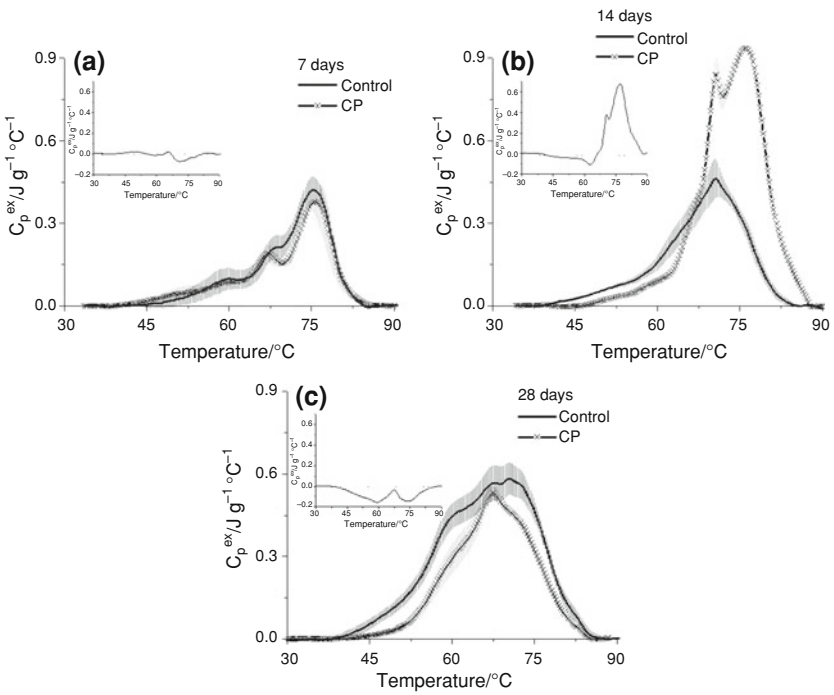

Fig. 4 DSC thermal transitions of blood serum from 7- (**a**), 14- (**b**), and 28-day old (**c**) newborn rats whose mothers were treated with cyclophosphamide (CP) in comparison with the control group. The shaded area is the standard deviation at each temperature. Inserts present differentiated curves between CP and the control groups

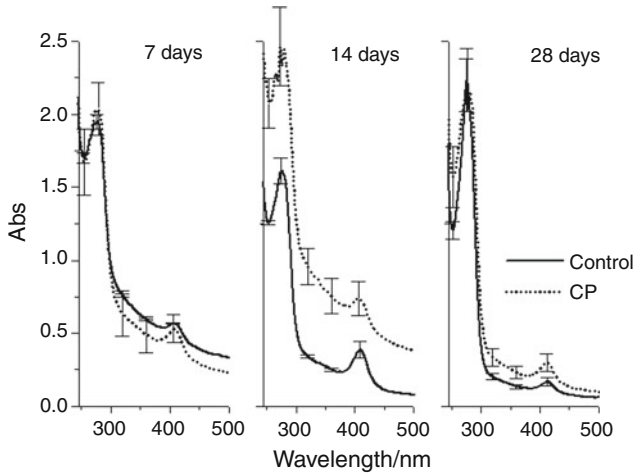

Fig. 5 Averaged absorption spectra of blood serum for control and cyclophosphamide (CP) group of 7-, 14-, 28-day old newborn rats

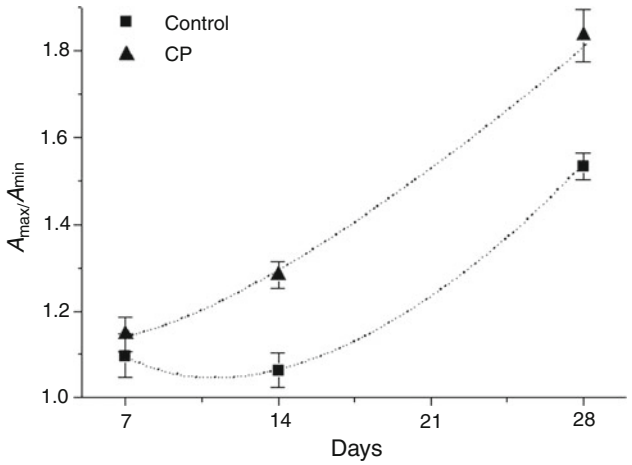

Fig. 6 The UV–VIS spectral ratio A_{max}/A_{min} (A_{max} local maximum at 278 nm, A_{min} local minimum at 252 nm) for control and cyclophosphamide (CP) group as a function of newborn rat's age

peptides with the plasma proteins than from changes in the content of these proteins.

Our results demonstrate that maternal administration of CP causes statistically essential disturbance in enthalpy changes (ΔH) (Fig. 3): an increase for 14- and decrease for 28-day rats in comparison with the control. Moreover, the values of the first moment (M_1) of the thermal transition presented in Table 1 confirm the difference between the control and CP groups. For serum samples from 14-day rats increase of M_1 is statistically essential and is still remarkable for the oldest newborns. It is noteworthy that the obtained values of M are close to the temperature of the peak maximum. It is dominated by globulin fractions and this fact suggests that these proteins are especially sensitive to CP administration as was previously mentioned in [23].

Sensitivity of serum proteins to cyclophosphamide application is also visible in UV–VIS absorption spectroscopy. For better insight into the problem the ratio A_{max}/A_{min} (A_{max} local maximum at 278 nm, A_{min} local minimum at 252 nm) was analyzed as a function of the rat's age (Fig. 6). Such a parameter was used by Artyukhov et al. [24] and Michnik et al. [25] as an indicator of structural changes induced in albumins by UV radiation. In our experiment the applied drug is just thought to be an external factor which can cause modifications in the molecular structure of serum proteins. There are statistically essential differences in age dependences of A_{max}/A_{min} ratio between the CP and control groups. They differ in slope and speed of increase. These changes may be connected with alternation in content of serum proteins as well as with the conformational reorganization of regions in serum proteins in which tryptophan and tyrosine are located.

An attempt was made to resolve the complex blood serum spectrum in the nearultraviolet region using the

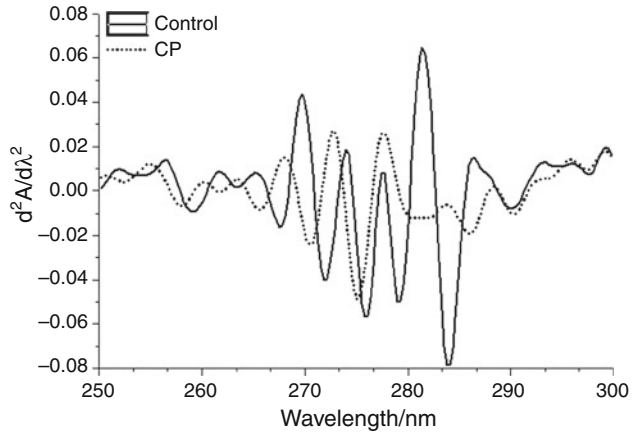

Fig. 7 Second derivative of absorption spectra for blood serum from 28-day newborn rats for control and cyclophosphamide (CP) group

second derivative methods. Figure 7 shows second derivative absorption spectra for blood serum obtained from 28-day newborn control and CP group. The well-resolved minima for control samples centred in the range of about 250–265 nm can be attributed to the phenyloalanine content. The remaining bands above 270 nm arise from the tyrosine and tryptophan residues [26]. In the first region (250–265 nm) the shifts of minima caused by maternal administration of CP are mainly observed. Above 270 nm not only shifts but also changes in intensities of the peaks are noted. It is interesting that the minimum at about 284 nm (Trp, Tyr) disappears after maternal administration of cyclophosphamide what can be due to the fact that alterations in protein proportions as well as in the microenvironment of amino acids can induce distortions in the protein molecular architecture [13].

Conclusions

The obtained results have shown that the thermal transition of newborn rat's blood serum proceed in the wide temperature range from 40 to 85 °C similarly as in human blood serum/plasma due to the analogous content of abundant serum proteins. However, the denaturation processes differ in their pathways. The heat effect from unfolding globulin forms seems to exceed that process connected with unliganded albumins. Thermal profiles and absorption spectra of blood serum are sensitive to the age of newborns as well as to effect of maternal administration of cyclophosphamide. The DSC measurements indicated the disturbance in the equilibrium of thermal denaturation of individual proteins within serum observed particularly in the case of samples from 14-day rats. It seems that the observed changes can be associated both with the alternation in protein content and the binding interactions in blood serum.

Our results confirm a potential usefulness of DSC and UV–VIS methods for diagnostic and monitoring changes with age as well as the pathological state of blood serum.

References

1. Sohár G, Pallagi E, Szabó-Révész P, Tóth K. New thermogravimetric protocol for the investigation of normal and damaged human hyaline cartilage. J Therm Anal Calorim. 2007;89:853–6.
2. Wiegand N, Vamhidy L, Patczai B, Domse E, Kereskai L, Lorinczy D. Differential scanning calorimetric examination of the human skeletal muscle in a compartment syndrome of the lower extremities. J Therm Anal Calorim. 2009;98:177–82.
3. Garbett NC, Mekmaysy CS, Helm W, Jenson AB, Chaires JB. Differential scanning calorimetry of blood plasma for clinical diagnosis and monitoring. Exp Mol Pathol. 2009;86:186–91.
4. Garbett NC, Millery JJ, Jenson AB, Chaires JB. Calorimetry outside the box: a new window into the plasma proteome. Biophys J. 2008;94:1377–83.
5. Michnik A, Drzazga Z, Michalik K, Barczyk A, Santura I, Sozańska E, Pierzchała W. Differential scanning calorimetry study of blood serum in chronic obstructive pulmonary disease; 2010. doi:10.1007/s10973-009-0602-6.
6. Pincus MR, Brandt-Rauf PW. Protein structure and cancer—cancer investigation. New York: Informa Healthcare; 1986. p. 185–95.
7. Lopez MF, Mikulskis A, Kuzdzal S, Bennett DA, Kelly J, Golenko E, DiCesare J, Denoyer E, Patton WF, Ediger R, Sapp L, Ziegert T, Lynch Ch, Kramer S, Whiteley GR, Wall MR, Mannion DP, della Cioppa G, Rakitan JS, Wolfe GM. High-resolution serum proteomic profiling of Alzheimer disease samples reveals disease-specific, carrier-protein-bound mass signatures. Clin Chem. 2005;51:101946–54.
8. Kochanek, Murphy SL, Anderson RN, Scott C. National vital statistics report. 2004; p. 53.
9. Koren G, Lishner M, Farine D. Cancer in pregnancy: maternal and foetal risk. Cambridge: Cambridge University Press; 1996.
10. Arnon J, Meirow D, Lewis-Roness H, Ornoy A. Genetic and teratogenic effects of cancer treatments on gametes and embryos. Hum Reprod Update. 2001;7:394–403.
11. Chabner BA, Longo DL. Cancer chemotherapy and biotherapy: principles and practice, 2nd edn. Lippincott-Raven, New York; Lippincott Williams & Wilkins; 2005.
12. Pratt WB, Ruddon RW. The anticancer drugs. Oxford: Oxford University; 1994.
13. Short R, Gibson J. Protein half-lives in neonatal mice after a toxic dose of cyclophosphamide. Cell Mol Life Sci. 1974;30:397–9.
14. Raichenok TF. Luminescence of human blood serum in the longwave spectral region. J Appl Spectrosc. 1999;66:470–2.
15. Mothé CG, Carestiato T, Águila MB. Thermoanalytical investigation of blood. J Therm Anal Calorim. 2006;2:247–51.
16. Kosa T, Maruyama T, Otagiri M. Species differences of serum albumins: II. Chemical and thermal stability. Pharm Res. 1998; 15:449–54.
17. Michnik A, Michalik K, Kluczewska A, Drzazga Z. Comparative DSC study of human and bovine serum albumin. J Therm Anal Calorim. 2006;84:113–7.
18. Michnik A, Drzazga Z. Thermal denaturation of mixtures of human serum proteins. J Therm Anal Cal. 2010; doi:10.1007/s10973-010-0826-5.
19. Michnik A, Drzazga Z, Michalik K, Kluczewska A. Differential scanning microcalorimetry study of the thermal denaturation of haemoglobin. Biophys Chem. 2005;118:93–101.
20. Jordan SM, Morgan EH. Albumin, transferrin and gamma-globulin metabolism during lactation in the rat. Q J Exp Physiol Cogn Med Sci. 1967;52:422–9.
21. Heim WG. The serum proteins of the rat during development. J Embryol Exptl Morphol. 1961;9:52–9.
22. Dobrowolski P, Śliwa E. The weaning-related changes in amino acids status of blood plasma in piglets. J Pre-Clin Clin Res. 2008; 2:071–4.
23. Mackiewicz U. Effects of cyclophosphamide on serum proteins in the rat. Acta Pol Pharm. 1967;24:549–53.
24. Artyukhov VG, Pantyavin AA, Vashanov GA. Vacuum-UV-radiation-induced structural-functional changes in serum albumin molecules. J App Spectrosc. 2001;68:291–8.
25. Michnik A, Michalik K, Drzazga Z. Effect of UVC radiation on conformational restructuring of human serum albumin. J Photochem Photobiol B. 2008;90:170–8.
26. Balestrieri C, Colonna G, Giovane A, Irace G, Servillo L. Second-derivative spectroscopy of proteins: studies on tyrosyl residues. Eur J Biochem. 1978;90:433–40.