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RP-HPLC determination of paraoxonase 3 activity in human blood serum

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Abstract

The aim of the present work was to establish conditions for paraoxonase 3 (PON3) activity determination in human blood serum with simvastatin (SV) as a substrate. The activity of PON3 is considered as a good early predictor of susceptibility to premature atherosclerosis as well as of statin therapy effectiveness. The method used quantifies the SV and β,δ-dihydroxyacid simvastatin (SVA) liberated from SV after incubation with blood serum, followed by deproteinization of the reaction mixture. Separation of SV and SVA was performed on an LC(18) column by isocratic elution with acetonitrile-K-phosphate buffer of pH 4.5 (v/v, 70:30) as a mobile phase at flow rate of 1.5 ml min^{-1}. Detection based on ultraviolet absorption at a wavelength of 239 nm was reliable for the simultaneous assay of SV and SVA. The applied method was sufficiently sensitive, precise and accurate for determination of low simvastatin lactone hydrolase (statinase) activity in blood serum of children (1.97–6.86 pmol $min^{-1}ml^{-1}$). The method is characterized by good linearity over the measurement range of 0.5–6 μg ml^{-1} (1.194–14.3 nmol ml^{-1}). Limits of detection (LOD) and quantitation (LOQ) for SV were 3.1 and 10.4 ng ml^{-1}, respectively. In case of SVA, LOD and LOQ were 4.7 and 14.44 ng ml^{-} for a 20 μl sample, respectively. Precision and accuracy of PON3 statinase activity determination in human blood serum with SV as substrate were satisfactory and acceptable for bioanalytical methods

Keywords: Statinase; Paraoxonase 3; PON3; HDL antioxidative enzymes

1. Introduction

Human paraoxonase 3 (PON3) and (PON1) are calcium-dependent esterases, closely associated with antiatherogenic action of blood serum high-density lipoproteins (HDL). PON3 is superior to PON1 as regards protection of LDL from oxidation, and is not inhibited by oxidized lipids. It can prevent formation of potentially atherogenic mildly oxidized LDL (mmLDL) and inactivate preformed mmLDL. PON3 may provide a basal constitutive atheroprotective function, whereas the protective effect of PON1 is more variable, in as much as PON1 expression is repressed by proatherogenic stimuli [1]. In contrast to PON1, PON3 exhibits no paraoxon hydrolase activity and very small, if any, arylesterase activity [2,3]. It has appeared that PONs are lactonases/lactonizing enzymes. Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and have distinctive substrate specificities. PON3 can hydrolyze bulky drug substrates such as lactones: mevastatin, lovastatin, spironolactone, 5- or 6-member ring and lactones with aliphatic substituents [4]. For many years it has been considered that PON1 is the source of statinase activity in human blood plasma [5], but in 2004, Draganov et al. found that its activity was the result of small amounts of PON3 in the purified PON1 preparation [6]. Statinase activity in blood serum is regarded as a biochemical indicator of constitutional antioxidative capacity of HDL and a determinant of some pharmacological actions of lactone statins. Simvastatin (SV), an analogue of lovastatin, is the lactone form of 1′,2′,6′,7′,8′,8a′-hexahydro-3,5-dihydroxy-2′,6′-dimethyl-8′(2″,2″-dimethyl-1″-oxobutoxy)-1′-naphthalene-heptanoic acid (SVA) Upon hydrolysis SV is converted to the β,δ-dihydroxy acid form (SVA), a potent competitive inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase [7]. The beneficial effects of statins may extend beyond improving the lipid profile [8]. Statinase can change pleiotropic action of SV [9]. Lactone itself does not exert hypocholesterolemic action. Statins containing the lactone ring in their molecules were the most potent coronary vasodilators [10], antiproliferative agents on smooth muscle cells [11,12] and inhibitors of proliferation of human breast

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cancer cells [13]. Lactones are up to 10-fold stronger inhibitors of multidrug resistance-associated transporters than the corresponding β,δ-dihydroxy acid form [14]. The interconversion between the lactone and acid forms will potentially influence drug-transporter interactions, and may ultimately contribute to the interindividual differences in pharmacokinetic profiles and therapeutic effects observed between statins [15]. The determination of statinase activity of PON3 seems to be a good early predictor of susceptibility to premature atherosclerosis as well as a predictor of statin therapy effectiveness. Determination of statinase activity in blood serum was not possible by direct UV spectrophotometric method, because there is no difference between spectra of the substrate (SV) and the product (SVA). Many chromatographic methods have been reported for SV and SVA determination: micellar electrokinetic chromatography [16], fluorescence detection, after derivatization to highly sensitive compounds for sensitive and selective analysis of SV and SVA [17] highly sensitive and selective methods gas chromatography-mass spectrometry (GC/MS) [18] with complicated clean procedure before assay, and liquid chromatography coupled with tandem mass spectrometry (LC-MS) [19,20]. However, a simple, selective, sensitive and reproducible method is needed for the assay of PON3 in blood serum samples. The only method we could apply in our clinical laboratory was the cost-effective method without derivatization, without gradient and with UV detection. HPLC methods with UV detection employed reversed-phase chromatography were simpler than other [21]. Unfortunately, the recommended, soundly validated method with UV detection, described by the European Pharmacopoeia was the gradient method [22]. We did not succeed in the isocratic elution method according to Billecke et al. [5], thus we decided to test the method of Godoy et al. [23], developed for stability studies of simvastatin drug substance and drug products.

The aim of the present work was to establish conditions for PON 3 simvastatin lactone hydrolase activity determination in human blood serum with SV as a substrate and the simultaneous determination of SV and SVA in the reaction mixture by liquid chromatography with ultraviolet absorbance detection.

2. Materials and methods

2.1. Materials

HPLC eluents: methanol and acetonitrile (both for HPLC, gradient grade) were obtained from J.T. Baker–(Deventer-Holland) and Merck (Darmstadt, Germany), respectively. Buffer components: ortho-phosphoric acid 85%, dipotassium hydrogen orthophosphate 3-hydrate and sodium hydroxide (all HiPerSolv) were obtained from BDH (Poole, UK). Tris(hydroxy-methyl)-aminomethane (analytical grade) was purchased from Fluka - BioChemika (Germany). Calcium chloride (analytical grade) was obtained from POCh-Gliwice, Poland. Serostandard (pooled lyophilized serum) was acquired from PTH HYDREX (Poland). Simvastatin (2,2-dimethylbutanoic acid (4R,6R)-6-[2[1S,2S,6R,8S,8aR)-1,2,6,7,8,8a-hexahydro-2,6-dimethyl-1-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl) ethyl]-1-napthalenyl ester] was obtained from Merck Sharp & Dohme (USA). SVA was prepared by alkaline hydrolysis in 0.02 mol l^{-1} NaOH, according to [5]. Water was obtained from Easy Pure RF compact ultra pure water system of Barnstead (resistivity: 18.3 MΩ cm^{-1}). Blood serum from slim or obese children after overnight fasting was used (after deep-freezing at −80 °C and thawing just before the beginning of the assay).

2.2. Standards

2.2.1. Standard solutions

SV stock solution I: 1 mg of simvastatin in 1 ml of methanol for HPLC was used to preparation of standard of SVA during alkaline hydrolysis.

SV stock solution II: 0.5 mg of simvastatin in 1 ml of methanol for HPLC, was used as a substrate for determination of PON3 activity and to preparation of SV working standard solution.

SV working standard solution (0.005 mg ml^{-1}): 10 μl of SV stock solution II was diluted ad 10 ml in methanol for HPLC.

SVA working solution (∼0.005 mg ml^{-1}): 100 μl of SVA solution, prepared by alkaline hydrolysis in 0.02 mol l^{-1} NaOH, according to [5] was neutralized and diluted ad 10 ml in methanol for HPLC. The solution (about 0.005 mg SVA ml^{-1} was introduced to the column (using of 20 μl sample loop) everyday as external standard. All stock solutions were stored at −20 °C. Working solutions were freshly prepared each day and stored at 4 °C until used.

2.2.2. Preparation of SVA

We have not a pure standard of SVA. Lactones proved to be unstable at alkaline pH, and thus: 1′,2′,6′,7′,8′,8a′-hexahydro-3,5-dihydroxy-2′,6′-dimethyl-8′(2″,2″-dimethyl-1″-oxobutoxy)-1′-naphthalene-heptanoic acid (SVA) was prepared in our laboratory by alkaline hydrolysis of SV. To 1 volume of *SV stock solution I*, 1 volume of 0.02 mol l^{-1} sodium hydroxide was added. The mixture was incubated during different time periods (30, 60, 90, 120, 150 min and 24 h) at 50 °C. Aliquots (100 μl) were removed at specified times, neutralized, by 0.02 mol l^{-1} HCl, mixed with a vortex, capped and stored under nitrogen at −20 °C until HPLC analysis. The optimum hydrolysis time was selected according to maximum area and height of SVA peak. Concentration of SVA released during hydrolysis was calculated from amount of decreasing SV.

2.3. PON3 activity determination

The basal reaction mixture: 940 μl of 20 mmol l^{-1} Tris–HCl (pH 7.6) containing 0.9 mmol l^{-1} $CaCl_2$ and 40 μmol l^{-1} of physostygmine was preincubated for 10 min at 37 °C in water-bath, then 10 μl of *SV stock solution II* was added (0.5 mg ml^{-1} of methanol). The final SV concentration in the assay mixture was 5 μg ml^{-1} (12 nmol ml^{-1}). The reaction was initiated by adding 50 μl of blood serum (except the blank one). After the proper time (1 h in a standard assay), the enzymatic reaction was stopped by deproteinization by cold acetoni-

trile (ACN). To achieve better deproteinization, the container with vials was then placed in an ice-bath for 5 min. Precipitated proteins were centrifuged for 6 min at maximum speed (14,000 rpm) at MPW-340 centrifuge (Fine Mechanic-Warsaw, Poland). The supernatants were poured into new glass tubes, the tubes were capped and stored in a refrigerator on ice, until HPLC analysis.

In a preliminary assays the deproteinization was performed in two ways: (A) by adding 1 ml of cold ACN or (B) by high-temperature treatment (vials were fast introduced into a boiling water-bath for 2 min and shaked). To study the optimum time for the enzymatic reaction, time periods from 1 to 200 min were applied (1, 20, 40, 60, 80, 10, 120, 140, 160, 180 and 200 min, respectively). SV and SVA quantitation in the deproteinized incubation mixture was performed using an isocratic HPLC method. PON3 activity was estimated from the decrease in SV and increase in SVA content in the incubation mixture (during 1 h of the enzymatic reaction).

2.3.1. Enzyme activity units and protein determination

The PON3 activity in blood serum was expressed as (pmol min^{-1} ml^{-1} of serum) and also as the specific activity of enzyme in blood serum (fmol $min^{-1}mg^{-1}$ of blood serum protein). The protein in a serum sample was measured by the biuret method [25].

2.4. High-performance liquid chromatographic determination of SV and SVA

HPLC analysis of SV and SVA was carried out using a Shimadzu series chromatograph consisting of a LC-10AD *Vp* pump, SPD-10AV *Vp* detector and manual Rheodyne valve. SV and SVA separations were performed on a Phenosphere-LUNA C18 (2), 5 μm, 250 mm × 4.60 mm column, at room temperature 22–25 °C. Peak detection was performed at λ = 239 nm.

2.4.1. Assay conditions

Twenty microliters of deproteinized incubation mixtures were introduced to the column. Separation of SV and SVA was performed by isocratic elution with acetonitrile – (0.03 mol l^{-1}) K-phosphate buffer of pH 4.5 (v/v, 70:30) as a mobile phase – at a flow rate of 1.5 ml min^{-1}. Separations were performed at room temperature (22–25 °C). A methanolic *SV working standard solution* and *SVA working solution* were used as external standards. The standard solutions were introduced at the beginning and at the end of each series of measurements.

2.4.2. Calibration curve

Blank serum samples were spiked with seven various working solutions of SV to obtain final concentrations: 0.5–6 μg ml^{-1} (1.19–14.30 nmol ml^{-1}). Calibration curves for simvastatin cover the concentrations of SV used for calculation of PON3 activity in human blood serum (up to 10 nmol ml^{-1}). Incubation mixtures for calibration curves were prepared in three variants, i.e. without serum and with ACN (A), with serum, after ACN protein precipitation (B), and with serum, after thermic protein precipitation (C). The SV calibration curves were obtained by plotting the peak area versus the nominal concentrations. The relevant equations were fitted by linear regression (the least squares method).

2.4.3. Validation of the method

Precision and accuracy of the method were evaluated by repetitive analysis of incubation mixtures (as for PON3 determination) spiked SV. For evaluation of the intra-day precision and the accuracy, the investigated mixtures contained blood plasma samples spiked at seven various concentration levels of SV ($n = 4$). To evaluate the inter-day precision and the accuracy, repetitive analysis of the blood plasma samples spiked at the lowest (LOQ), medium and high concentration levels of calibration samples was performed ($n = 6$). The intra-day precision and accuracy data were obtained from the analysis of the above-mentioned samples spiked SV on 1 day by the same operator. The inter-day precision and accuracy data were obtained by assaying samples prepared at three levels of concentration on different days by two operators. The method of SVA determination in reaction mixture was not done, since a pure standard of SVA is essential for its validation.

3. Results and discussion

At the present time PON3 is widely considered the only enzyme catalyzing the hydrolysis of lactone ring of statins. Other esterases of blood serum such as pseudocholinesterase do not have such qualities. Liquid chromatography method elaborated by Godoy et al. [23], for fast determination of simvastatin in drug substance, proved to be good for the determination of PON3 in human blood serum.

3.1. UV detection of SV and SVA after HPLC separation

Using an isocratic HPLC elution, we were able to determine the substrate (SV) and the product (SVA) of enzymatic reaction catalyzed by paraoxonase 3 during a single run of analysis. The chromatographic method avoids a problem connected with the lack of difference between spectra of SV and SVA. The simultaneous determination of SV and SVA in the appropriate PON3 incubation mixture requires only deproteinization and introduction of supernatant on a chromatographic column. Application of the buffered mobile phase containing phosphate buffer pH 4.5 slows down the hydrolysis of the lactone during analysis. At pH between 4.0 and 6.0, it is practically not hydrolyzed [23] and because of that repeatability of assay results was better, than in more acidic phase. Application of Phenosphere-LUNA C18 (2), 5 μm, 250 mm × 4.6 mm column provides good separation of SV and SVA at room temperature 22–25 °C. The use of Phenosphere-LUNA C18 column instead of Chromolith Performance RP-18e monolithic column (100 mm × 4.6 mm), as was in Godoy investigations [23], results in longer retention times. The chromatograms of SV and SVA in the plasma samples of the children are shown in Fig. 1B–D. They do not show any interfering peak at the retention time similar to that of SV

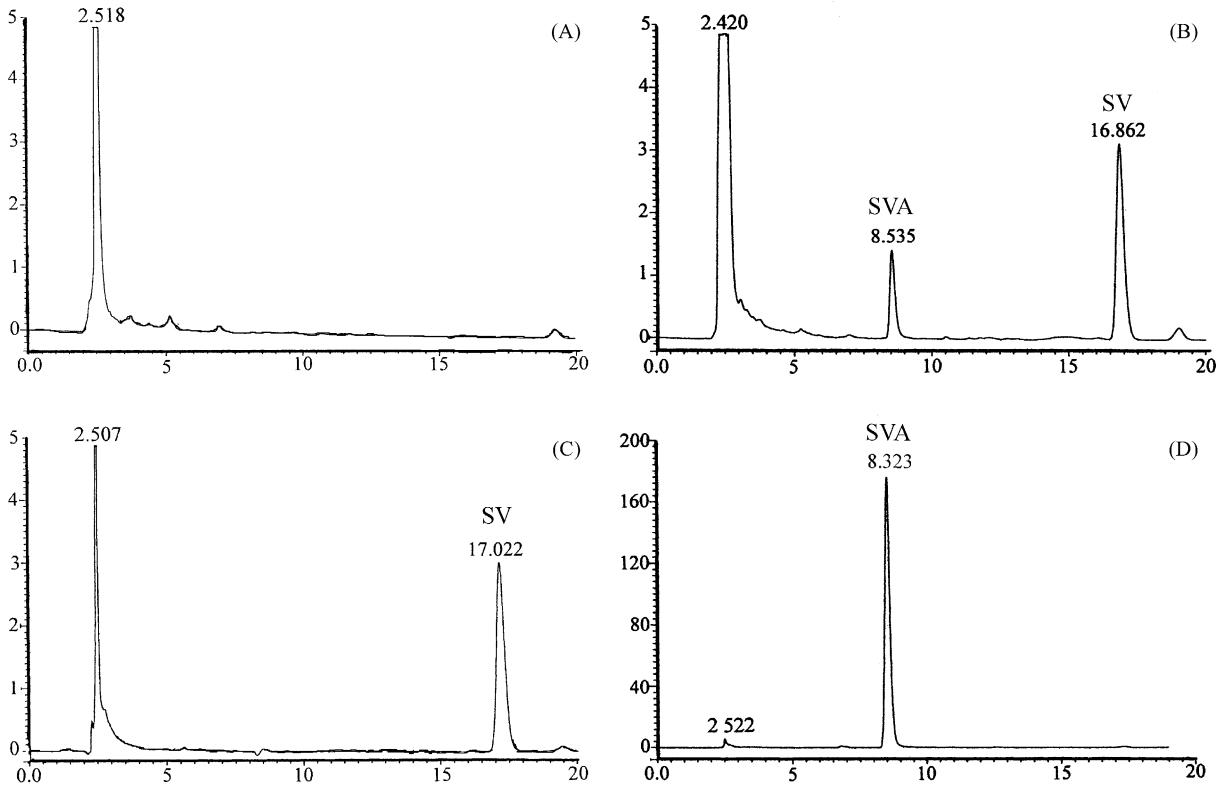

Fig. 1. Chromatograms of blank blood serum (A) sample after termination of the PON3 reaction (B), sample of serum supplemented with SV (C), and the product of alkaline hydrolysis of SV (D). SV—simvastatin, SVA—β,δ-hydroxy acid, product of SV hydrolysis.

and/or SVA in blood plasma (Fig. 1A). The average retention time for SVA was 8.3 min and for SV 17.1 min (8.32 ± 0.06 min and 17.05 ± 0.23 min, respectively).

3.1.1. Linearity of the method of SV in blood serum determination

The assay was validated by analyzing seven SV standards. As a criterion of the linearity of the method, a correlation coefficient of the corresponding curve determined by the least squares method was used. The method applied to the determination of SV during PON3 activity assay showed a good degree of linearity. The linear relationship was observed within the concentration range from 0.5 to 6 μg ml^{-1} (1.194–14.3 nmol ml^{-1}). The corresponding linear regression equations are presented in Table 1. The resulting calibration curves for simvastatin covered the full range of the measured concentrations and made it possible to read data obtained during determination of PON3 activity in blood serum of all the children under study. The initial concentration of SV in the incubation mixture for PON3 determination was 12 nmol ml^{-1} (5 μg ml^{-1}). PON3 activity (in 50 μl of blood serum) induced a decrease of 3–5 nmol ml^{-1} during 1-h incubation. After 1-h incubation, the most concentrations of remained SV were found in the middle of the curve. The corresponding linear regression equation is presented in Table 1 (columns B and C). The resulting equations were used to calculate the concentrations of SV in the test sample.

Table 1
Linearity parameters of calibration curve $y = ax + b$ at λ = 239 nm

Parameter	*A* (without serum and with ACN) ($n = 6$)	*B* (with serum after ACN deproteinization) ($n = 6$)	*C* (with serum after thermic deproteinization) ($n = 6$)
a	12702	11963	11478
b	6106	8891	9847
S_a	316.8	333.3	361.45
S_b	2634.5	2771.8	3005.8
r	0.998	0.998	0.997

(*a*) Slope, (*b*) intercept, (S_a) mean square error, (S_b) mean square error, (*r*) correlation coefficient.

3.1.2. Accuracy and precision of the method of SV in blood serum determination

The accuracy and precision of the method were determined from the variation of the standards from the regression line. The precision of the method was defined as the closeness of agreement between independent test results obtained under conditions described for PON3 activity determination. It was expressed in terms of relative standard deviation (R.S.D.) of the determined concentrations. The accuracy of the method was defined as the closeness agreement between the test results and the accepted reference value and is expressed as (%bias). The intra-day precision and accuracy for the measurement of SV in human serum are shown in Table 2. The %bias for each calibration level showed good accuracy of calibration curves. The intra-day precision (R.S.D.) for the SV calibration standard ranged from 3.4% to 5.2% after ACN deproteinization, and from 4.4% to 8.9% after high temperature deproteinization. The accuracy

Table 2
Intra-day precision and accuracy of SV measurements in human serum after deproteinization with ACN or incubation in boiling water

Concentration, spiked [μg ml^{-1}]	Deproteinization with acetonitrile ($n=4$)			Thermic deproteinization ($n=4$)		
	Concentration, found [μg ml^{-1}]	Accuracy (%)	Precision (R.S.D.%)	Concentration, found [μg ml^{-1}]	Accuracy (%)	Precision (R.S.D.%)
0.5	0.487 ± 0.021	97.4	4.3	0.493 ± 0.042	98.6	8.6
1	0.986 ± 0.049	98.6	5.0	1.089 ± 0.0767	108.9	7.1
1.5	1.57 ± 0.072	105.0	4.6	1.507 ± 0.108	100.5	7.2
2.5	2.583 ± 0.135	103.3	5.2	2.332 ± 0.208	93.3	8.9
3.75	3.56 ± 0.125	95.1	3.5	3.5481 ± 0.155	94.6	4.4
5	4.68 ± 0.164	93.5	3.5	4.671 ± 0.233	93.4	5.0
6	5.69 ± 0.192	94.8	3.4	5.512 ± 0.289	91.9	5.2

for the SV calibration standard ranged from 93.5% to 105% after ACN deproteinization, and from 91.9% to 108.9% after high-temperature deproteinization. The intra-day precision and accuracy was better after ACN deproteinization. These results showed that the method of SV determination is linear from 0.5 to 6 μg ml^{-1} (a range 2 to 5 μg ml^{-1} is needed for enzyme activity calculation). An internal standard was not added to each assay due to good precision of the data. To provide a reliable control of SV and SVA determinations, we introduced as external standards: one standard point for SV (5 μg ml^{-1}) and one standard point for SVA to each of series of determinations (∼5 μg ml^{-1}).

The inter-day precision and accuracy were determined by analyzing three control pools on 3 consecutive days. The inter-day precision and accuracy for the measurement of SV in human serum are shown in Table 3. The precision (R.S.D.) for the SV calibration standard at the lowest (LOQ), medium and the highest concentration levels of calibration samples amounted to 8.8%, 7.1% and 4.1%, respectively, when ACN was used to deproteinization. After the thermic deproteinization, the values were similar (7.1%; 10.1% and 3.9%, respectively). The inter-day precision of both method deproteinization was similar, but the accuracy was worse after thermic deproteinization, than after ACN deproteinization. The detected concentrations after ACN deproteinization were (104% vs. 88%), (95.4% vs. 131%) and (102% vs. 129%), respectively.

3.1.3. Limit of detection and limit of quantitation

UV detection of SV and SVA at 239 nm is highly sensitive. Limits of detection (LOD) and quantitation (LOQ) for SV were 9.3 and 16.3 ng ml^{-1}, respectively, for a 20 μl sample. For SVA, LOD and LOQ were 4.7 and 14.4 ng ml^{-1}, respectively. For SVA, LOD and LOQ were 0.14 and 0.5 ng ml^{-1}, respectively.

3.2. Optimalization of the enzymatic reaction conditions

3.2.1. Choice of deproteinization method

The calibration curves of SV after protein precipitation with ACN or high-temperature treatment have acceptable accuracy and precision, although thermic deproteinization reduces precision at low and medium concentrations (Table 2). Also, thermic method of deproteinization significantly worsened the accuracy of assay (the average accuracy was 88% for low and about 130% for medium and high concentrations at inter-day measurements). The use of boiling bath temperature can be the reason for the overestimation of SV in the reaction mixture (Table 3), probably because of the increase of non-enzymatic and enzymatic hydrolysis of SV, before temperature-induced enzyme denaturation takes place. High temperature can also accelerate the formation of SV oxidized products [24]. Because of that, in later enzymatic hydrolysis studies only cold ACN was applied as a deproteinizing reagent.

3.2.2. Alkaline hydrolysis of SV-preparation of a working solution of SVA

Alkaline hydrolysis of SV provided the source of working solution of SVA for further determination of SVA (a product of enzymatic reaction catalyzed by PON3). SVA was identified on the basis of retention time in HPLC separation. After 2 h hydrolysis only one dominating peak was observed (Fig. 2A). The time course curve of SVA formation exhibits satisfying linearity up to 120 min and shows that the long-term hydrolysis (over 120 min) is associated with a considerable decrease in SVA formation (Fig. 3). It also reveals presence of new small peaks on the chromatogram resulting (the difference between SVA and contaminations are presented in the chromatogram enclosed) (Fig. 2B). These results suggest that high temperature

Table 3
Inter-day precision and accuracy of SV measurement in human serum after deproteinization with ACN or incubation in boiling water ($n=6$)

Concentration, spiked [μg ml^{-1}]	Deproteinization with acetonitrile ($n=6$)			Thermic deproteinization ($n=6$)		
	Concentration, found [μg ml^{-1}]	Accuracy (%)	Precision (R.S.D.%)	Concentration, found [μg ml^{-1}]	Accuracy (%)	Precision (R.S.D.%)
0.5	0.520 ± 0.088	104	8.8	0.442	88.4	7.1
2.5	2.390 ± 0.071	95.4	7.1	3.270	130.8	10.1
5	5.083 ± 0.0413	101.7	4.1	6.432	128.67	3.9

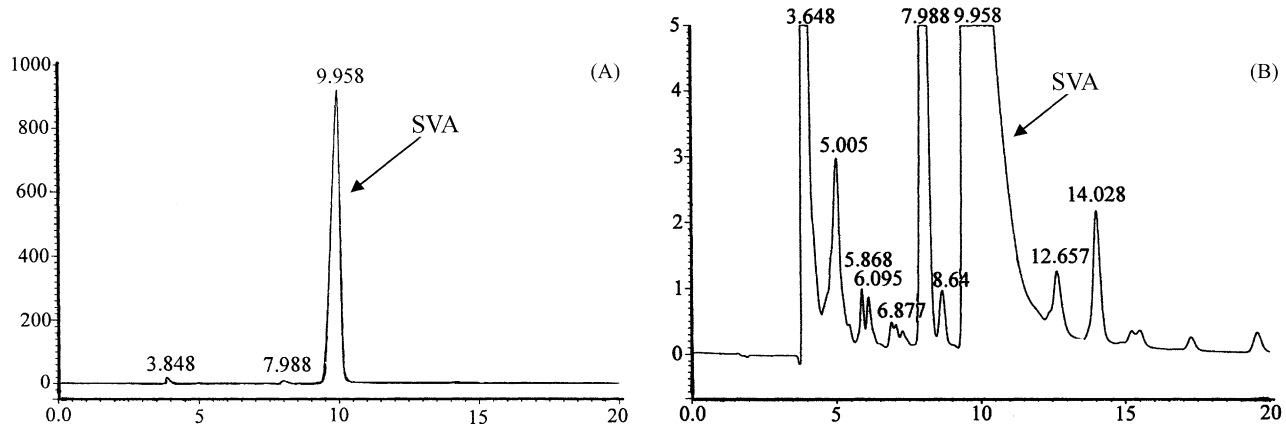

Fig. 2. Chromatogram of concentrated sample after 120 min of alkaline hydrolysis of SV. Chromatogram is presented at various UV detector sensitivities: (A) at 1000 mV, (B) at 5 mV.

of hydrolysis (50 °C) and oxygen athmosphere can accelerate the oxidative degradation of SV and/or SVA. The susceptibility of statins to oxidative degradation is well established [24,25]. During the investigations of lovastatine (demethylated analog of simvastatin) stability, Javernik et al. stated that the amount of lovastatin, after exposing the sample to an oxygen atmosphere, decreased about 40%, but the content of impurities did not reach the level of the degraded lovastatin [26]. They suggest that the volatile oxidation products, and the products without chromophores (doubly bound in naphthalene ring) result from the oxidation processes. It is possible that simvastatin in our experiments was subjected similar, but not as profound degradation. If so, the purity of the standard solution of SVA is not perfect, and because of that we have not conducted the validation procedure for SVA (the quantity of SVA in an HCl-neutralized solution was recalculated from a decrease in SV concentration during alkaline hydrolysis). The initial concentration of SV was 1190 nmol ml^{-1}, after 2 h the concentration of SVA reaches 720 nmol ml^{-1}. The product of alkaline hydrolysis neutralized, divided into small portions was kept in nitrogen-filled and well-capped vials at −20 °C. It was used for preparation of SVA working solution introduced each day on the column at the beginning and at the end of assay series, to control retention time value and resolution of the peaks.

3.2.3. Choice of incubation time for PON3 determination

Determination of an extent of the product (SVA) formation with respect to incubation time (Fig. 4) was performed at a substrate concentration lower than Km (Michaelis constant), because of poor substrate solubility in water environment (the incubation mixture). The enzymatic reaction catalyzed by blood serum PON3 was linear up to 200 min when only simvastatin hydrolysis was monitored and was linear only up to 80 min when SVA release was monitored (effect similar to that observed dur-

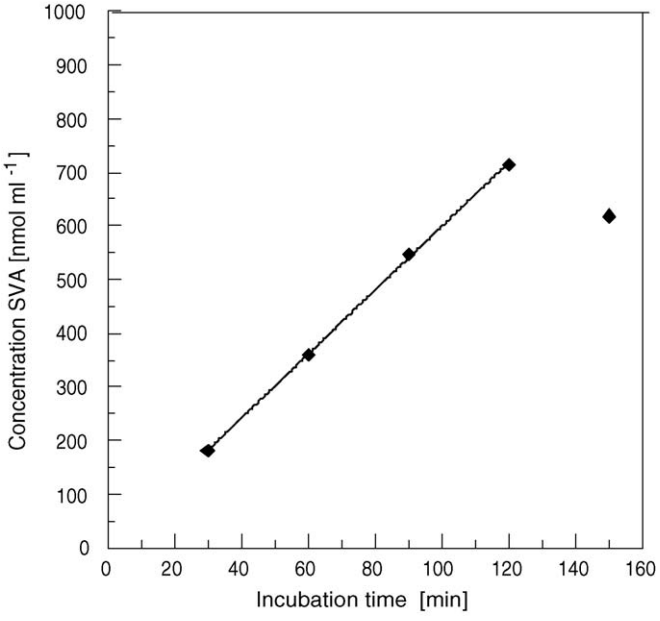

Fig. 3. Change of SVA concentration during alkaline hydrolysis of SV. Each value was obtained from three experiments.

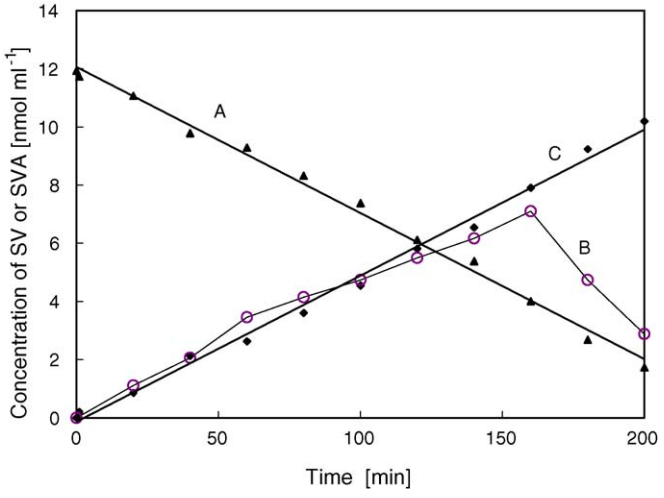

Fig. 4. Product (SVA) formation versus time of SV enzymatic hydrolysis. Curve (A): Change of SV concentration during enzymatic hydrolysis estimated by HPLC. Curve (B): Change of SVA concentration estimated by HPLC. Curve (C): Change of SVA concentration calculated as difference between initial SV concentration and current concentration of SV estimated by HPLC. Each value was obtained from three experiments.

ing alkaline hydrolysis). The reason for the decrease of SVA concentration in reaction mixture is not yet known. It had not yet been found whether what causes it is actually the oxidative degradation of SVA or whether new metabolites of SV and/or SVA actually form. Because of a low velocity of the reaction and limited linearity of SVA releasing, the time of the enzymatic reaction should be between 30 and 60 min. The incubation time selected for preliminary assays of PON3 in human blood serum was fixed as 60 min. Judging on the peak area and the height of the peak, we suggest that incubation time at 37 °C can be made even shorter.

3.3. Enzyme activity in blood serum

Blood serum statinase, which could hydrolyze statins in vitro has to be present in antiatherogenic high density of lipoproteins (HDL) fraction in order to interact with lipophylic substrate of the reaction it catalyses. Previously considered it could be PON 1, Draganov et al. using GFC method found that PON3 is the only enzyme capable of catalyzing the hydrolysis of lactone ring of statins in antiatherogenic HDL fraction [6]. Unfortunately, no specific inhibitors for paraoxonases activities were known. Such inhibitor should confirm that PON3 is the only source of statinase activity in our reaction mixture. Other esterases of blood serum such as pseudocholinesterase do not have such activities. However, just in case, the incubation mixture for the determination of PON3 activity also contains 40 μmol/l of physostygmine—an inhibitor of cholinesterase activity.

The mean PON3 (simvastatine-lactonase) activity determined in blood serum of slim and obese children was 2.42 ± 0.64 (pmol min^{-1} ml^{-1} of serum) and 3.87 ± 1.26 (pmol min^{-1} ml^{-1} of serum), respectively. The specific activity of PON3 in the slim and obese children was: 36.58 ± 6.88 (fmol min^{-1} mg^{-1} of blood serum proteins) and 50.75 ± 18.86 (fmol min^{-1} mg^{-1} of blood serum proteins), respectively. The average inter-day coefficient of variation (CV) for enzyme activity was found to be 4.57% (calculated from SV decrease during the PON3 catalyzed reaction, for 4 consecutive days) and 7.31% when the activity was measured once a week for 6 weeks, respectively. The presented data show that the precision of enzymatic determination of PON3 as simvastatine lactonase is good and acceptable for bioanalytical methods [27]. The assay of PON3 in human blood serum with the use of RP-HPLC method creates new perspectives for diagnostic application of this interesting enzyme as early indicator of susceptibility to arteriosclerosis and indicator of potential drug side effects. Induction of the PON3 catalyzed hydrolysis of statin lactones to their respective hydroxy acids could also result in an increase in the plasma AUC of statin hydroxy acids and should be considered as a potential mechanism of the observed gemfibrozil-statin pharmacokinetic interaction and severe side effects of statins [28].

4. Conclusions

The validated method of PON 3 in blood serum determination is simple, not very expensive, gives reliable findings, and has a chance to be introduced to clinical laboratory. This method is characterized by satisfactory linearity, good precision and accuracy of the substrate determination, in spite of its very low concentration in the blood serum. The main advantage of this method is that the substrate and the product of the enzymatic reaction can be determined during the same run of analysis and using the same mobile phase. This makes it possible to perform current clinical interpretation and quality control of laboratory determinations. The determination of statinase activity of PON3 using this method seems to be a good early predictor of susceptibility to premature atherosclerosis and a predictor of statin and spironolactone therapy effectiveness.

References

[1] C.J. Ng, D.M. Shih, S.Y. Hama, N. Villa, M. Navab, S.T. Reddy, Free Radic. Biol. Med. 38 (2005) 153–163.
[2] S.T. Reddy, D.J. Wadleigh, V. Grijalva, C. Ng, S. Hama, A. Gangopadhyay, D.M. Shih, A.J. Lusis, M. Navab, Al.M. Fogelman, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 542–547.
[3] D.I. Draganov, P.L. Stetson, C.E. Watson, S.S. Billecke, B.N. La Du, J. Biol. Chem. 275 (2000) 33435–33442.
[4] D.I. Draganov, J.F. Teiber, A. Speelman, Y. Osawa, R. Sunahara, B.N. La Du, J. Lipid Res. 46 (2005) 1239–1247.
[5] S. Billecke, D. Draganov, R. Counsell, P. Stetson, C. Watson, C. Hsu, B.N. La Du, DMD 28 (2000) 1335–1342.
[6] D.I. Draganov, B.N. La Du, Pharmacogen. Naunyn Schmiedebergs Arch. Pharmacol. 369 (2004) 78–88.
[7] S. Vickers, C.A. Duncan, I.W. Chen, A. Rosegay, D.E. Duggan, DMD 8 (1990) 138–145.
[8] E. Rosati, N. Aracri, A. Bottone, C. Cau, E. Scotti, Minerva Cardioangiol. 50 (2002) 63–68.
[9] M. Werner, J.M. Sacher, Br. J. Pharmacol. 143 (2004) 715–724.
[10] R.J. Gryglewski, W. Uracz, J. Swies, S. Chlopicki, E. Marcinkiewicz, M. Lomnicka, J. Madej, Ann. NY Acad. Sci. 947 (2001) 229–245.
[11] A.K. Van Vliet, P. Negre-Aminou, G.C.F. van Thiel, P.A. Bolhuis, L.H. Cohen, Biochem. Pharmacol. 52 (1996) 1387–1392.
[12] P. Negre-Aminou, M. van Erck, R.E. van Leeuwen, J.G. Collard, Biochem. Pharmacol. 61 (2001) 991–998.
[13] A.O. Mueck, H. Seeger, D. Wallwiener, Menopause 10 (2003) 332–336.
[14] C. Chen, R.J. Mireles, S.D. Campbell, J. Lin, J.B. Mills, J.J. Xu, T.A. Smolarek, DMD 33 (2005) 537–546.
[15] T. Prueksaritanont, Y. Qiu, L. Mu, K. Michel, J. Brunner, K.M. Richards, J.H. Lin, Pharm. Res. 22 (2005) 1101–1109.
[16] M.K. Srinivasu, A.N. Raju, G.O. Reddy, J. Pharm. Biomed. Anal. 29 (2002) 715–721.
[17] H. Ochiai, N. Uchiyama, K. Imagaki, S. Hata, T. Kamei, J. Chromtogr. B 694 (1997) 211–217.
[18] X.-S. Miao, C.D. Metcalfe, J. Chromtogr. A 998 (2003) 133–141.
[19] H. Yang, Y. Feng, Y. Luan, J. Chromtogr. B 785 (2003) 369–375.
[20] N. Zhang, A. Yang, J.D. Rogers, J.J. Zhao, J. Pharm. Biomed. Anal. 34 (2004) 175–187.
[21] G. Carlucci, P. Mazzeo, L. Biordi, M. Bologna, J. Pharm. Biomed. Anal. 10 (1992) 693–697.
[22] European Pharmacopoeia, Supl. (2001) 1403–1405.
[23] R. Godoy, C.G. Godoy, M. De Diego, C. Gómez, Chil. Chem. Soc. 49 (2004) 289.
[24] M.J. Kaufman, Pharm. Res. 7 (1990) 289–292.
[25] S. Javernik, S. Kreft, B.F. Strukelj, Pharmazie 56 (2001) 738–740.
[26] S.R. Javernik, S. Kreft, T. Borut, V. Franc. Croat. Chem. Acta 76 (2003) 263–268.
[27] S. Braggio, R.J.P. Grossi, M.A. Cugola, J. Pharm. Biomed. Anal. 14 (1996) 375–388.
[28] T. Prueksaritanont, J.J. Zhao, J.J.B. Ma, B.A. Roadcap, C. Tang, Y. Qiu, L. Liu, J.H. Lin, P.G. Pearson, T.A. Baillie, J. Pharmacol. Exp. Ther. 301 (2002) 1042–1051.