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Enantioselective binding analysis of verapamil to plasma lipoproteins by capillary electrophoresis–frontal analysis

Niveen Abdel Latif Mohamed[a,1], Yukihiro Kuroda[a], Akimasa Shibukawa[a,*], Terumichi Nakagawa[a], Samia El Gizawy[b], Hassan F. Askal[b], Micheal E. El Kommos[b]

[a]*Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-Ku, Kyoto-shi 606-8501, Japan*
[b]*Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Assiut University, Assiut, Egypt*

Abstract

Capillary electrophoresis coupled with frontal analysis was applied to the study of enantioselective binding of verapamil (VER) to plasma lipoproteins. The drug–lipoprotein mixed solution, which had been in the binding equilibrium, was hydrodynamically introduced into a non-coated fused-silica capillary. Since VER is positively charged in the neutral run buffer (pH 7.4), the unbound VER enantiomers migrated toward the cathodic end much faster than negatively charged lipoproteins and their bound forms. Once unbound VER migrated apart from lipoprotein, the bound VER was quickly released from the protein to maintain the binding equilibrium. Thus, VER migrated as a zone through the capillary and gave a trapezoidal peak with a plateau region on the electropherogram. The VER concentration in this plateau region was equal to the unbound VER concentration in the initial sample solution. It was found that the bindings of VER to high-density lipoprotein (HDL), low-density lipoprotein (LDL) and oxidized LDL were not site-specific and not enantioselective. Partition-like binding to lipid part of these lipoproteins seemed to be dominant. The total binding affinities of LDL to VER were about seven-times stronger than those of HDL, and the oxidation of LDL by copper ion enhanced the binding affinities significantly. © 2000 Published by Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Frontal analysis; Binding studies; Lipoproteins; Proteins; Verapamil

1. Introduction

A drug in plasma is in the state of variable and complicated protein binding equilibrium. The bound drug is difficult to penetrate into blood vessel wall and cell membrane, whereas the unbound drug can pass through the blood capillary wall to reach the target-site as well as to undergo metabolism. Therefore, plasma protein binding of a drug significantly affects its pharmacokinetic and pharmacodynamic properties [1–3]. Several plasma proteins such as albumin, α_1-acid glycoprotein (AGP) and lipoproteins can contribute to the plasma protein binding of a drug simultaneously, and therefore the overall protein binding property in whole plasma can be described as the sum of respective protein binding properties. Therefore, quantitative study of binding to the individual plasma protein is necessary to

*Corresponding author. Tel.: +81-75-753-4531; fax: +81-75-753-4578.
E-mail address: akimasas@pharm.kyoto-u.ac.jp (A. Shibukawa)
[1]Present address: Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

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understand in detail the plasma protein binding of the drug.

About 50% of chiral drugs are clinically used as racemate, whereas it is often the case that either one of enantiomers shows pharmaceutical activity. Because the difference in the protein binding property between the enantiomers often causes the difference in their pharmacokinetic characters [4,5], enantioselective protein binding study is essential for the development of new racemic drugs and for the safety in their clinical use.

Plasma lipoproteins are the major transporter of cholesterol and triglycerides in the blood stream. Plasma lipoproteins also act as the transporter of drugs; several hydrophobic and/or basic drugs are known to be bound to plasma lipoproteins [6]. Their concentrations in plasma vary depending on disease state such as coronary artery disease, and the inter-individual difference is also often observed. Such variable plasma levels of lipoproteins may give a significant change in the plasma distribution of drugs. Lipoprotein is a meta-stable molecular aggregate which consists of lipophilic core (cholesterol ester and triglycerides) surrounded by a surface layer including polar lipids (phospholipids+free cholesterol) and apolipoproteins. Because apolipoproteins as well as lipid components such as cholesterol, cholesterol ester and some phospholipids are chiral compounds, the binding between lipoproteins and a chiral drug may show enantioselective property. However, enantioselective binding study of lipoproteins has not been investigated.

Plasma lipoproteins are classified into several subclasses depending on the density. Among these subclasses, high-density lipoprotein (HDL) and low density lipoprotein (LDL) are most important because of higher plasma concentrations than others. LDL suffers from in vivo oxidation. Oxidized LDL is known to play an important role in atherogenesis by direct cytotoxicity, by chemotactic effect on monocytes, by an inhibitory effect on macrophage motility and by initiation of foam cell formation by macrophages leading to the formation of atherosclerotic plaques that take up oxidized LDL via their scavenger receptors [7–10].

Drug–protein binding affinity has been incorporated into capillary electrophoresis (CE) systems as a driving force of specific drug analysis. For example, albumin [11], α_1-acid glycoprotein, ovomucoid, conalbumin [12,13] and ovoglycoprotein [14] have been used as a chiral selector in chiral CE separations. On the contrary, drug–protein binding affinity can be quantitatively estimated by the separation systems such as high-performance liquid chromatography (HPLC) and CE. Several methods have been proposed for the drug–protein binding study, such as frontal analysis, the Hummel–Dreyer method, affinity HPLC using immobilized protein, affinity CE and the vacancy peak method [15,16]. So far, we have developed and applied high-performance capillary electrophoresis–frontal analysis (HPCE–FA) to the analysis of plasma protein binding study [17–20], and investigated the effect of AGP glycan structures upon the enantioselective AGP–verapamil (VER) binding [19,20].

VER, a calcium channel blocker, is clinically used as a racemate, although the (*S*)-isomer shows much stronger pharmacological activity [21]. In the present study, we applied HPCE–FA to investigate the binding of VER enantiomers to plasma lipoproteins. Because long-time preservation and large-scale preparation of lipoproteins are difficult, HPCE–FA which allows rapid binding analysis with a small sample injection volume (ca. 100 nl) is beneficial to the binding study of lipoproteins. It is reported that VER has anti-oxidant activity against oxidation of LDL [22–24]. However, the binding affinity between VER and oxidized LDL has not been reported. In this paper, the effect of LDL oxidation upon the binding to VER was also investigated.

2. Materials and methods

2.1. Materials and apparatus

HPCE–FA analysis was done in one of the following CE systems, CAPI 3000 (Otsuska Electronics, Japan) and 270A (Applied Biosystems, CA, USA). The former system was equipped with an uncoated fused-silica capillary of 57 cm (45 cm effective length)×75 μm I.D. The latter system was equipped with a Z-shape type uncoated fused-silica capillary of 122 cm (100 cm effective length)×75 μm I.D. (LC Packings, CA, USA) which provides with about 40-times longer light path for detection

than a usual capillary. A Beckman L7-65 was used for ultracentrifugation. A UV–Vis spectrophotometer UV-1200 and spectrofluorophotometer RF-5300PC (Shimadzu, Japan) were used to monitor the oxidation of LDL. Standard materials of (*R*)- and (*S*)-verapamil hydrochloride were obtained from Research Biochemicals (Natick, MA, USA). The purity (>99%) of each enantiomer was confirmed by chiral HPCE under conditions described in Ref. [18]. The drug–protein mixed solutions were made up in sodium phosphate buffer (pH 7.4, ionic strength 0.17). Bovine serum albumin (fatty acid-free) was purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of HDL and LDL

Human HDL and LDL were prepared from plasma of a healthy male volunteer by sequential ultracentrifugation [25]. Briefly, human plasma, the density of which was 1.006 g/ml, was ultracentrifuged for 24 h at 50 000 rpm at 4°C, and the upper fraction was removed. The density of the remainder was adjusted to 1.063 g/ml, and the following ultracentrifugation (20 h at 38 000 rpm, 4°C) gave the LDL fraction (upper fraction). The density of the lower fraction was adjusted to 1.21 g/ml, and the ultracentrifugation (48 h at 38 000 rpm, at 4°C) gave the HDL fraction (upper fraction). In the above procedure, the density was adjusted using NaBr. The NaBr concentration in the plasma sample (density= 1.063 g/ml) was about 0.5 *M*, and that in the plasma sample (density=1.21 g/ml) was about 1.5 *M*. The HDL fraction (density, 1.063~1.21 g/ml) and LDL fraction (density, 1.006~1.063 g/ml) were further purified by size-exclusion HPLC. The HPLC conditions were as follows: column, HiLoad Superdex 200 pg (60 cm×2.6 cm I.D., Pharmacia); mobile phase, sodium phosphate buffer (pH 7.4, ionic strength, I=0.17); flow-rate, 2 ml/min; column temperature, 4°C; detection, UV at 254 nm. The purified HDL and LDL fractions were concentrated on the membrane (Centriplus-10, Amicon).

2.3. Oxidation of LDL

LDL was oxidized by Cu^{2+} [26]. $CuSO_4$ was added to the LDL fraction up to 5 μ*M*. Then the LDL fraction containing copper was shaken gently for 12 h at 37°C. After shaking, the copper ion was removed by washing the precipitate repeatedly with a sodium phosphate buffer (pH 7.4, I=0.17) at 4°C using the Centriplus-10 (Amicon). The oxidation was monitored by the UV absorption at 234 nm and by the fluorescence intensity (excitation 360 nm, emission 430 nm) [27].

2.4. Determination of lipoprotein concentrations

The concentrations of lipoproteins were calculated as follows. First, the concentration of apolipoprotein was measured by the modified Lowry method [28] in which sodium dodecyl sulfate (SDS) solutions were used to prevent the interference of lipid components. Bovine serum albumin (fatty acid-free) was used as the standard protein in preparing the calibration curve. The molar concentration of lipoprotein was then estimated assuming that the apoprotein content and the molecular mass of HDL are 50% (w/w) and $1.8\cdot10^5$, and those of natural and oxidized LDL are 21% (w/w) and $2.3\cdot10^6$, respectively [6].

2.5. Determination of unbound VER concentrations by HPCE–FA

The drug and lipoprotein were mixed in run buffer solution (sodium phosphate buffer, pH 7.4, I=0.17). The mixed solution was hydrodynamically introduced into the capillary for 4 s (ca. 100 nl) or 6 s (ca. 150 nl), and a positive voltage (+7 kV) was applied to start electrophoresis. The VER peak was monitored by UV at 205 nm. The temperature was set at 25°C. Positively charged unbound VER migrated faster than the negatively charged lipoprotein and the bound form. Because drug–protein binding is reversible and kinetically rapid, their binding equilibrium is kept unchanged during separation from lipoprotein. As a result, there appears a zone of unbound drug which is detected as a trapezoidal peak having a plateau zone. Thus the unbound drug concentration can be measured from the plateau height. If the electrophoretic velocity of free protein is widely different from that of drug–protein bound form, the drug concentration in the plateau zone will become different from the original unbound drug concentration, because the binding equilibrium changes during the electrophoretic separation process [29].

However, this problem is negligible so far as the binding does not bring about a considerable change in the protein mobility, as in case of warfarin–albumin binding [30]. Our preliminary studies also showed that the bindings of propranolol and nilvadipine to HDL and LDL did not change the electrophoretic velocity of these lipoproteins. Therefore, we neglected the change in the velocity of proteins due to binding to VER. After each binding analysis, the capillary was washed with 30 m*M* SDS and run buffer each for 1 min. It is reported that a rinse of the inner capillary surface by SDS solution is useful to improve separation efficiency and to achieve reproducible analyses of samples containing plasma samples [31,32].

As mentioned later, the binding of VER enantiomers to lipoproteins shows non-specific and partition-like character. It is well known that the total binding affinity (nK) of non-specific binding can be estimated by the following equation,

$$\mathrm{nK} = (C_\mathrm{T} - C_\mathrm{u})/(C_\mathrm{u} C_\mathrm{P})$$

where C_T represents the total drug concentration in the sample solution, C_u represents the unbound drug concentration measured by the present method, and C_P represents the concentration of lipoprotein in the sample solution, respectively.

A series of the standard drug solutions [10~100 μ*M* (*R*)- or (*S*)-VER in phosphate buffer, pH 7.4, $I = 0.17$] without containing protein were used to prepare calibration curves. The calibration curves thus obtained indicated good linearity ($R > 0.999$).

3. Results and discussion

Fig. 1 shows the typical electropherograms of (*R*)-VER. Fig. 1A shows the electropherogram of VER in protein-free sample solution, and hence the plateau height represents the total drug concentration. On the other hand, Fig. 1B and C show the electropherograms of (*R*)-VER in native LDL solution and in the oxidized LDL solution, respectively, at the same drug concentration as in the protein-free solution. The plateau height became obviously lower than that of the protein-free sample solution due to protein binding. The unbound drug concentration was determined from this plateau height. Almost the same electropherograms were obtained in the analyses of samples containing (*S*)-VER.

At first, the effect of sample injection time was investigated. 40 μ*M* (*S*)-VER and 14.6 μ*M* HDL mixed solution was analyzed under different injection time (4, 6 and 8 s). While the VER peak width became broader with increasing injection time, the plateau height was almost unchanged regardless of the injection time, and the unbound VER concentration was determined as 28.7±0.16 μ*M*, 27.8±1.62 μ*M* and 29.0±1.52 μ*M* ($n = 2$ or 3) for the injection times of 4, 6 and 8 s, respectively. This means that these injection times are long enough to verify the applicability of frontal analysis. Thus, in the following analyses, the injection time was set at 4 or 6 s.

Since lipoprotein is a molecular aggregate of apolipoproteins and lipid components, two different binding modes would be possible. One is the binding between drug and apolipoprotein, which would be site-specific like in case of albumin and α_1-acid glycoprotein. Another is the binding to lipid components, which would be non-specific and partition-like. These different binding modes can be distinguished from the relation between unbound drug fraction and total drug concentration. If the unbound

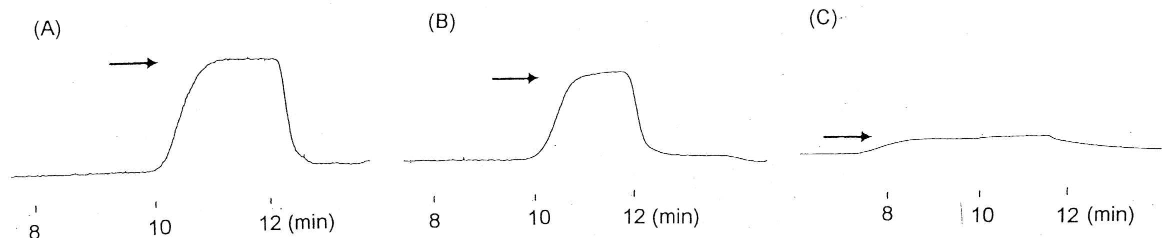

Fig. 1. HPCE–FA of VER–LDL binding. Sample, (A) 50 μ*M* (*R*)-VER without LDL, (B) 50 μ*M* (*R*)-VER + 1.90 μ*M* LDL, (C) 50 μ*M* (*R*)-VER + 1.90 μ*M* oxidized LDL. CE conditions: equipment, CAPI-3000. Capillary, 30 (effective length) + 22 cm × 75 μm I.D. Run buffer, sodium phosphate buffer (pH 7.4, $I = 0.17$). Applied voltage, +7 kV. Detection, UV at 205 nm. Injection volume, 6 s.

drug fraction is increased with increasing total drug concentration, the non-specific binding mode is dominant. On the other hand, in case the binding is non-saturable and the unbound drug fraction is independent of the total drug concentration, the non-specific partition-like binding mode is dominant.

Fig. 2 shows the relation between unbound VER fraction and the total VER concentration in HDL solution. The total concentration was increased from 25 μ*M* to 100 μ*M*, while HDL concentration was kept constant at the physiological level (14.6 μ*M*). It is found in Fig. 2 that the unbound fraction is independent of the total drug concentration. The major protein constituents in HDL are apolipoprotein A-I and A-II. It is estimated that one HDL contains five to six molecules of these apolipoproteins on average [33]. Therefore, the concentration of these A apolipoproteins in the sample solutions is estimated as 73–88 μ*M* in total. As shown in Fig. 2, VER–HDL binding was kept constant even when the total drug concentration became higher than the apolipoprotein concentration. This result indicates that VER–HDL binding is non-specific. In addition, the unbound fractions of both enantiomers were almost equal each other, and virtually no enantioselectivity was observed.

Fig. 3 shows the relation between unbound VER fraction and the total VER concentration in native and oxidized LDL solutions, where the total concentration was increased from 40 μ*M* to 100 μ*M*, while LDL concentration was kept at the physiological level (1.90 μ*M*). Similarly to the binding to HDL

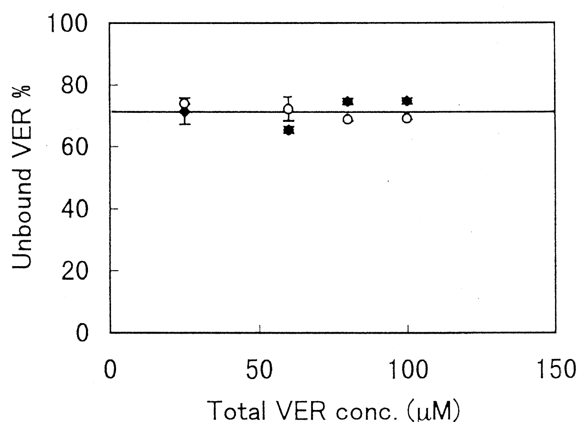

Fig. 2. Relation between total concentration and unbound fraction of (*R*)-VER (♦) and (*S*)-VER (○) in 14.6 μ*M* HDL solutions.

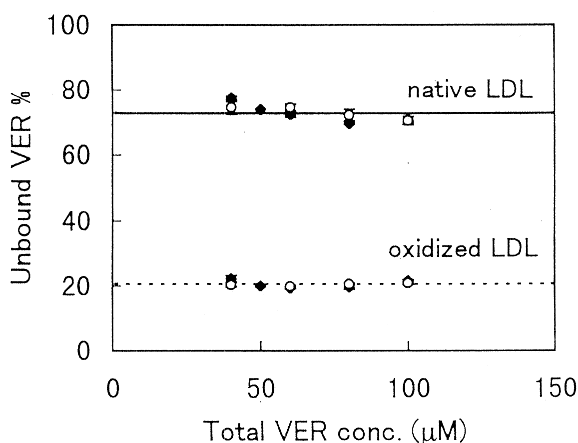

Fig. 3. Relation between total concentration and unbound fraction of (*R*)-VER (♦) and (*S*)-VER (○) in 1.90 μ*M* native or oxidized LDL solutions.

shown in Fig. 2, the unbound VER fraction in LDL solution was also unchanged regardless of the total drug concentration. Apolipoprotein B is the major protein constituent in LDL particle, and one molecule of apolipoprotein B exists per one LDL particle [34]. In this study, the drug binding was not saturated even when the total drug concentration was twenty times or much higher than the apolipoprotein concentration. This indicates that, same as in VER–HDL binding, the VER–LDL binding is also non-specific. In addition, the unbound fractions of both enantiomers are almost equal to each other, indicating no significant enantioselectivity in VER–LDL binding.

The LDL oxidation gives rise to the change in UV absorption at 234 nm due to the formation of conjugated diene in the lipid phase [35]. It also derives the increase in fluorescence intensity (excitation 360 nm, emission 430 nm) due to Schiff base formation by reaction of ε-amino group of lysine residue in apolipoprotein B with aldehydes or hydroperoxides which are the degradation products from lipid peroxidation of unsaturated fatty acids composed of esterified cholesterol and phosphatidylcholine [36]. After 12 h oxidation by copper ion, the UV absorbance of 0.019 μ*M* LDL solution increased from 0.095 to 0.187, and fluorescence intensity changed from 1.57 to 13.2 (arbitrary units). This means that both lipids and apolipoprotein suffered from this oxidation. The electrophoretic mobility of

LDL increased from -0.0118 cm^2/min·V to -0.0136 cm^2/min·V, which indicates increase in negative net charge.

The mixed solutions containing 40~100 μM VER and 1.90 μM oxidized LDL were analyzed by the present methods, and the unbound drug fractions were calculated as shown in Fig. 3. As in case of native LDL, the unbound VER fraction was constant regardless of the total drug concentration, indicating non-saturable and partition-like binding character. Also, no significant enantioselectivity was observed. As clearly found from Fig. 3, the unbound VER fraction in the oxidized LDL is much lower than that in the same concentration (1.90 μM) of native LDL solution, which means that the binding affinity was increased by oxidation.

Table 1 lists the total binding affinity (nK) of VER enantiomers calculated from the data shown in Figs. 2 and 3. The affinity of VER–LDL binding is about seven-times stronger than that of VER–HDL, and the affinity was enhanced by about 10-times after oxidation of LDL.

In conclusion, HPCE–FA is a useful method for binding study of plasma lipoproteins. VER is bound to HDL, LDL and oxidized LDL in non-specific and partition-like manner, and no enantioselectivity was observed. These results suggest that the partition-like binding to the lipid part is dominant. LDL with higher lipid content shows stronger affinity to VER than HDL, and the oxidation of LDL increases the binding affinity by 10-fold. The present method allows micro-scale binding assay, and hence facilitates the binding study of plasma lipoproteins which cannot stand large scale preparation and long time preservation.

Table 1
Total binding affinity (nK) between VER enantiomers and lipoproteins[a]

	nK (M^{-1})	
	(R)-VER	(S)-VER
HDL	2.75 (±0.614)·10^4	2.81 (±0.326)·10^4
Normal LDL	1.99 (±0.307)·10^5	1.96 (±0.221)·10^5
Oxidized LDL	2.03 (±0.158)·10^6	2.06 (±0.077)·10^6

[a] Mean±SD (n=16).

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References

[1] M.C. Meyer, D.E. Guttman, J. Pharm. Sci. 57 (1968) 895.
[2] J.J. Vallner, J. Pharm. Sci. 66 (1977) 447.
[3] T.C. Kwong, Clin. Chem. Acta 151 (1985) 193.
[4] G.T. Tucker, M.S. Lennard, Pharmacol. Ther. 45 (1989) 309.
[5] T. Noctor, in: I.W. Wainer (Ed.), Drug Stereochemistry, 2nd ed., Marcel Dekker, New York, 1993, Chapter 12.
[6] S. Urien, in: J.P. Tillement, E. Lindenlaub (Eds.), Protein Binding and Drug Transport, F.K. Schattauer Verlag, Stuttgart, New York, 1986, pp. 63–75.
[7] D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo, J.L. Witztum, New Engl. J. Med. 320 (1989) 915.
[8] J.L. Witztum, D. Steinberg, J. Clin. Invest. 88 (1991) 1785.
[9] M.T. Quinn, S. Parthasarathy, L.G. Fong, D. Steinberg, Proc. Natl. Acad. Sci. 84 (1987) 2995.
[10] M.T. Quinn, S. Parthasarathy, D. Steinberg, Proc. Natl. Acad. Sci. 82 (1985) 5949.
[11] D.K. Lloyd, S. Li, P. Ryan, J. Chromatogr. A 694 (1995) 285.
[12] Y. Tanaka, S. Terabe, J. Chromatogr. A 694 (1995) 277–284.
[13] Y. Tanaka, S. Terabe, Chromatographia 44 (1997) 119–126.
[14] J. Haginaka, N. Kanasugi, J. Chromatogr. A 782 (1997) 281–288.
[15] J. Oravcova, B. Boehs, W. Lindner, J. Chromatogr. B 677 (1996) 1.
[16] D.S. Hage, S.A. Tweed, J. Chromatogr. B 699 (1997) 499.
[17] A. Shibukawa, Y. Yoshimoto, T. Ohara, T. Nakagawa, J. Pharm. Sci. 83 (1994) 616.
[18] T. Ohara, A. Shibukawa, T. Nakagawa, Anal. Chem. 67 (1995) 3520.
[19] H. Shiono, A. Shibukawa, Y. Kuroda, T. Nakagawa, Chirality 9 (1997) 291.
[20] Y. Kuroda, A. Shibukawa, T. Nakagawa, Anal. Biochem. 268 (1999) 9.
[21] H. Echizen, M. Manz, M. Eichelbaum, J. Cardiovasc. Pharmacol. 12 (1988) 543.
[22] M.I. Dushkin, Y.S. Schwarz, Biochem. Pharmacol. 49 (1995) 389.
[23] N. Rojstaczer, D.J. Triggle, Biochem. Pharmacol. 51 (1996) 141.
[24] I. Karmansky, N. Fruener, Clin. Chem. Acta 259 (1997) 177.
[25] F.T. Hatch, R.S. Lees, Adv. Lipid Res. 6 (1968) 1.
[26] B.J. Auerbach, C.L. Bisgaier, J. Wolle, U. Saxena, J. Biol. Chem. 271 (1996) 1329.

[27] R. Maeba, H. Shimasaki, N. Ueta, Biochim. Biophys. Acta 1215 (1994) 76.
[28] M.A.K. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, Anal. Biochem. 81 (1978) 206.
[29] M.H.A. Busch, J.C. Kraak, H. Poppe, J. Chromatogr. A 777 (1997) 329.
[30] M.H.A. Busch, L.B. Carels, H.F.M. Boelens, J.C. Kraak, H. Poppe, J. Chromatogr. A 777 (1997) 311.
[31] T. Nakagawa, Y. Oda, A. Shibukawa, H. Fukuda, H. Tanaka, Chem. Pharm. Bull. 37 (1989) 707.
[32] D.K. Lloyd, H. Watzig, J. Chromatogr. B 663 (1995) 400.
[33] G.M. Kostner, P. Laggner, in: J.C. Fruchart, J. Shepherd (Eds.), Human Plasma Lipoproteins, Walter de Gruyter, Berlin, New York, 1989, pp. 23–54.
[34] R.W. Mahley, T.L. Innerarity, S.C. Rall Jr., K.H. Weisgraber, J. Lipid Res. 25 (1984) 1277.
[35] C.E. Thomas, R.L. Jackson, J. Pharmacol. Exp. Ther. 256 (1991) 1182.
[36] U.P. Steinbrecher, J. Biol. Chem. 262 (1987) 3603.