LC-MS/MS Analysis of Canine Lipoproteins Fractionated Using the Ultracentrifugation-Precipitation Method

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ABSTRACT. Due to the lack of a gold standard method in canine lipoprotein analysis, it is unclear whether canine high-density lipoprotein (HDL) and low-density lipoprotein (LDL) can be accurately evaluated by the lipoprotein analysis methods used for dogs. This study investigated whether the ultracentrifugation-precipitation (U-P) method was suitable as a gold standard method for analyzing canine lipoprotein. First, the U-P method was compared with a gel permeation high-performance liquid chromatography system (GP-HPLC). The concentrations of canine HDL cholesterol (HDL-C) and LDL cholesterol (LDL-C) determined by the U-P method correlated closely with those determined by GP-HPLC. However, the canine HDL-C concentration determined by the U-P method was lower than that determined by GP-HPLC, and the canine LDL-C concentration determined by the U-P method was lower than that determined by GP-HPLC, and the canine HDL could be precipitated with heparin manganese chloride solution. Second, the HDL and LDL fractions separated by the U-P method were analyzed by LC-MS/MS. The HDL fraction was found to contain only apolipoprotein A-I, which is an apolipoprotein of HDL, whereas the LDL fraction contained both apolipoprotein A-I and apolipoprotein B-100, which is an apolipoprotein of LDL. This data showed that a certain lipoprotein that includes apolipoprotein A-I might precipitate with canine LDL when using heparin manganese chloride solution. These results indicated that the U-P method is not currently a gold standard method for analyzing canine lipoproteins. KEY WORDS: canine lipoprotein, LC-MS/MS, ultracentrifugation-precipitation method.

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Lipoproteins consist of proteins and lipids, and they transport lipids and cholesterol to many organs in the bloodstream. Human plasma lipoproteins are classified by specific density as chylomicrons (CM), very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). VLDL and LDL include apolipoprotein B-100 (apo B-100), and the HDL particle includes apolipoprotein A-I (apo A-I). Since altered balance of human lipoproteins is involved in arteriosclerosis, cardiovascular diseases and metabolic syndrome [4, 6, 7, 12], the importance of analyzing the human lipoprotein profile has been gaining recognition. In the area of veterinary medicine, the analysis of canine lipoproteins is not often utilized, because its clinical significance remains unclear [3, 10]. Few reports have addressed the analysis of canine lipoproteins. In addition, human lipoproteins separate perfectly according to density: the density of LDL particles ranges from 1.019 to 1.063 g/ml, and that of HDL particles ranges from 1.063 to 1.210 g/ml. Therefore, the ultracentrifugation method [9], whereby lipoproteins are separated by specific density, is the

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gold standard method for analyzing human lipoproteins. A gel permeation high-performance liquid chromatography system (GP-HPLC) [20] and polyacrylamide gel and agarose gel electrophoresis [8, 13] are used as methods for detailed examination of human lipoproteins. However, canine lipoproteins are not separated by their specific density, because the densities of canine HDL and LDL particles overlap [15]: canine LDL particles are detected in the wide density range of 1.006 to 1.087 g/ml, and canine HDL particles are detected in the range of 1.025 to 1.210 g/ml. Therefore, Barrie et al. [2] reported a combined ultracentrifugation and precipitation (U-P) method for fractionating the canine lipoproteins from canine plasma. Because canine VLDL, LDL and HDL were fractionated using the U-P method, it was supposed that this method could be appropriate for analyzing canine lipoproteins in canine plasma. In the report of Barrie et al. [2], canine apo A-I and apo B-100 were not analyzed using detailed methods. Currently, antibodies against canine apo A-I and apo B-100 are unavailable, meaning that immunological analysis is not possible for canine lipoproteins. The agarose gel electrophoresis method was utilized for analyzing canine lipoproteins in a few reports [1]. Recently, Mizutani et al. [17] reported that GP-HPLC would be useful in explication in veterinary practice. However, it remains unclear whether those methods can accurately separate canine HDL from canine LDL. Therefore, there is no gold standard method for analyzing canine lipoproteins. For this reason, the U-P method was examined for its suitability as a gold

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standard method for canine lipoprotein analysis. Puppione et al. [18] had reported detecting canine apo A-I, apolipoprotein A-II, proapolipoprotein A-I, apolipoprotein C-II and apolipoprotein A-IV in canine HDL using tandem mass spectrometry (MSMS), suggesting that mass spectrometry could be an efficient method for detecting canine apolipoproteins. Therefore, we employed liquid chromatography tandem mass spectrometry (LC-MS/MS) to perform detailed analyses of canine lipoproteins in this study. Moreover, it was supposed that specific antibodies against canine apo A-I and apo B-100 would not be necessary for detecting those canine apolipoproteins. In the present study, to examine whether the U-P method was suitable as a gold standard method for canine lipoprotein analysis, the canine HDL and LDL fractions obtained by the U-P method were analyzed in detail through LC-MS/MS.

MATERIALS AND METHODS

Dogs: Four adult male and 4 adult female beagle dogs were used in this study. It was carried out in accordance with Hokkaido University guidelines for the care and use of experimental animals, which basically conform to those of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The study was approved by the Committee for Laboratory Animals, Graduate School of Veterinary Medicine, Hokkaido University (approval number 08-0473).

The ultracentrifugation-precipitation method for canine lipoprotein analysis: The ultracentrifugation-precipitation (U-P) method for canine lipoprotein analysis was performed using the protocol of Barrie et al. [2] with some modifications. Briefly, blood samples were collected from dogs that had been fasted for 24 hr. Peripheral blood was taken into tubes with EDTA, and the plasma was separated by centrifugation at 1,500 \times g for 10 min at 4°C. Four milliliters of the plasma was placed in a thermoplastic ultracentrifuge tube and overlayered with 6 ml of 154 mM NaCl. The tube was capped and centrifuged at 164,000 \times g for 18 hr at 4°C in an ultracentrifuge (Beckman L7-55 Ultracentrifuge, Beckman Coulter Inc., Brea, CA, U.S.A.). Three milliliters of the upper layer, which included lipoproteins with a density of less than 1.006 g/ml(VLDL), were removed as the VLDL fraction. Seven milliliters of the lower layer containing lipoproteins with a density greater than 1.006 g/ml(HDL and LDL) were collected as the HDL+LDL fraction. The LDL in the HDL+LDL fraction was precipitated by incubation with 400 μl of 1 U/ μl heparin for 10 min and 5 ml of 101 mM MnCl₂ for 30 min on ice. After centrifugation at 10,800 \times g for 30 min at 4°C, the resultant supernatant was collected as the HDL fraction, and the sediment was collected as the LDL fraction.

Measurement of cholesterol concentration in fractions separated by the ultracentrifugation-precipitation method: The cholesterol concentrations of intact plasma (total cholesterol), the HDL+LDL fraction and the HDL fraction (HDLcholesterol, HDL-C) were measured using an enzymatic assay (Chorestest[®], Sekisui Medical Co., Ltd., Tokyo, Japan) with a spectrophotometer (U-3210, Hitachi, Tokyo, Japan). The LDL-cholesterol (LDL-C) concentration was calculated as the difference between the cholesterol concentration of the HDL+LDL fraction and that of the HDL fraction. For the U-P method, the LDL/HDL ratio was calculated from the LDL-C and HDL-C concentrations calculated above.

Analysis of canine lipoprotein using GP-HPLC: Lipoprotein analyses of intact plasma and the HDL+LDL and HDL fractions were performed using commercial GP-HPLC service (LipoTEST, Spectrum Lab. Japan Co., Ltd., Tokyo, Japan). The concentrations of HDL-C and LDL-C determined by the U-P method were compared with those determined by GP-HPLC. The LDL/HDL ratio of GP-HPLC was also calculated.

LC-MS/MS sample preparation by tryptic digestion: LC-MS/MS sample preparation was performed using the methods of Rosenfeld et al. [19] and Wilm et al. [21] with some modifications. Briefly, 7 microliters of the HDL+LDL, HDL and LDL fractions, separated using the U-P method. were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) with 4-20% gradient acrylamide gels (Ready Gel J, Bio-Rad Laboratories, Inc., Benicia, CA, U.S.A.) and stained with Coomassie Brilliant Blue [14]. The stained protein's bands were excised with a scalpel, washed with 100 *ul* of 50% acetonitrile in 25 mM ammonium carbonate and left to dry at RT for 10 min with shaking in an Eppendorf Thermomixer. The gel slices were dehydrated with 100 μl acetonitrile and dried in a vacuum centrifugal dryer (Thermo Savant SPD SpeedVac, with a Savant UVS 400 Universal Vacuum System, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Then, the gel slices were alkylated with 55 mM iodoacetamide and 25 mM ammonium carbonate in light interception with aluminum foil for 45 min. Thirty microliters of 10 µl/ml trypsin gold (Promega Corp., Madison, WI, U.S.A.) was added to the gel slices, and the gel slices were added to 50 mM ammonium carbonate and incubated on ice for 30 min. After absorption of the protease solution, the gel slices were incubated at 37°C for 12 hr. To extract hydrophilic and hydrophobic peptides from the gel slices, we performed a three-step extraction. The first extracted solution (50% acetonitrile and 5% trifluoroacetic acid [TFA]) was added to the gel slices and then incubated in an ultrasonic bath (USC-6, Osada, Inc., Los Angeles, CA, U.S.A.) for 10 min. Next, the second extracted solution (99% acetonitrile and 0.1% TFA) was added to the gel slices and incubated in an ultrasonic bath for 10 min. Finally, the third extraction (99.9% ultrapure water and 0.1% TFA) was added to the gel slices and incubated in an ultrasonic bath. These samples produced by the three-step extraction were concentrated to $\sim 20 \ \mu l$ in the vacuum centrifugal dryer.

LC-MS/MS analysis: The samples prepared above were analyzed by Fourier transform mass spectrometry (LTQ Orbitrap, Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) interfaced with the UltiMate 3000 Nano LC systems (Thermo Fisher Scientific Inc.) equipped with a trap column (Zorbax 300 SB-C18, 0.3×5 mm, Agilent Technology, Santa Clara, CA, U.S.A.) and analytical column (Acclaim PepMap100 3 μ m 100 Å, Dionex Corp., Sunnyvale, CA, U.S.A.) in ace-



tonitrile gradient (acetonitrile:water=70:30, v/v). Peak lists were generated from raw LC-MS/MS spectra using Xcalibur 2.0.7 (Thermo Fisher Scientific Inc.). The raw data acquired by the mass spectrometry experiment were searched against the National Center for Biological Information (NCBI) databases (www.ncbi.nlm.nih.gov) using Proteome Discoverer 1.0 (Thermo Fisher Scientific Inc.).

Statistical analysis: The correlation of the HDL-C and LDL-C concentrations between the U-P method and GP-HPLC was investigated by Pearson's correlation coefficient test. Results were considered as correlated when r was significantly different from 0 (P<0.05) and over 0.7.

RESULTS

Analysis of canine lipoproteins in fractions separated using the ultracentrifugation-precipitation method: First, intact canine plasma, the HDL plus LDL fractions and HDL fractions separated by the U-P method were analyzed using GP-HPLC. The peaks of VLDL, LDL and HDL cholesterol were identified in intact plasma, but no peak was observed for CM cholesterol, because of the 24 hr fast (Fig. 1A). The HDL+LDL fraction showed peaks for LDL and HDL cholesterol, but not for VLDL cholesterol (Fig. 1B). Moreover the HDL fraction showed only one peak of HDL cholesterol (Fig. 1C). Next, the concentrations of HDL and LDL cho-



Fig. 1. GP-HPLC of canine lipoprotein in intact dog plasma (A) and of the HDL+LDL fraction (B) and HDL fraction (C) separated by the U-P method. Solid line: Cholesterol concentration. This analysis was repeated three times. The figure is representative of the three experiments.

lesterol determined using the U-P method were compared with those determined by GP-HPLC. The canine HDL-C concentration determined by the U-P method correlated well (r=0.94), but was not the same as that determined by GP-HPLC (Fig. 2A). The concentrations of canine HDL-C determined by the U-P method were lower than those determined by GP-HPLC. The canine LDL-C concentration determined by the U-P method correlated well with that determined by GP-HPLC (r=0.93) (Fig. 2B). However, the concentrations of canine LDL-C determined by the U-P method were higher than those determined by GP-HPLC. The canine LDL/HDL ratio determined by the U-P method correlated with that determined by GP-HPLC (r=0.75), but was not the same as that determined by GP-HPLC (Fig. 2C).

SDS-PAGE of the canine HDL+LDL, HDL and LDL fractions: The canine HDL+LDL, HDL and LDL fractions separated by the U-P method were subjected to SDS-PAGE. It was found that the canine HDL+LDL, HDL and LDL fractions from 3 healthy dogs included several proteins, and the amounts of these proteins were different in each dog (Fig. 3). In the HDL+LDL and LDL fractions, proteins with an estimated molecular weight of greater than 250 kDa and of approximately 25 kDa were detected in all dogs (Fig. 3A and 3C). In the HDL fraction, proteins with an estimated molecular weight of approximately 25 kDa were detected in all dogs (Fig. 3B). In both the HDL and LDL fractions, proteins with





an estimated molecular weight of approximately 35 and 60 kDa were also detected (Fig. 3B and 3C).

LC-MS/MS analysis of the canine apolipoprotein A-I and apolipoprotein B-100: The proteins with a molecular weight of 25 kDa in the HDL fraction were analyzed using LC-MS/MS. The results of LC-MS/MS analysis are shown in Fig. 4. The proteins were identified significantly as apo A-I (Fig. 4). The proteins with a molecular weight of more than 250 kDa in the LDL fraction were also analyzed using the LC-MS/MS and identified significantly as canine apo B-100 (Fig. 5). The proteins with a molecular weight of 60 kDa were identified as canine albumin (data not shown). The proteins with a molecular weight of 35 kDa were identified as haptoglobin (data not shown).

DISCUSSION

The present study examined the U-P method for its suitability as a gold standard method for canine lipoprotein analysis. Since it was reported that GP-HPLC was an efficient method in small animals [17], the U-P method was compared with GP-HPLC first. In the U-P method, an ultracentrifuge separates VLDL particles by specific gravity, and a heparin manganese chloride solution precipitates LDL particles [2]. GP-HPLC separates the particles based on size by using a specialized column [17]. It was confirmed

Fig. 2. Correlation of HDL-C (A) and LDL-C (B) concentrations and LDL/HDL ratio (C) between the U-P method and GP-HPLC. Five beagle dogs were used in this study.

that VLDL particles were removed from canine plasma by ultracentrifugation and that LDL was removed from the HDL+LDL fraction by precipitation. Moreover, the HDL-C and LDL-C concentrations determined by the U-P method correlated well with those determined by GP-HPLC, although the results of the U-P method were not consistent with those of GP-HPLC. It was suggested that some of the canine HDL could be precipitated with heparin manganese chloride solution, resulting in a higher LDL-C concentration and lower HDL-C concentration in the U-P method than in GP-HPLC. Furthermore, the presence in canine plasma of an apolipoprotein E (apo E)-rich subfraction of HDL with a similar density to LDL and apo E, which may co-precipitate with LDL under certain conditions, may invalidate some of the methodologies used for the measurement of canine LDL-C and HDL-C concentrations [2]. Therefore, the HDL and LDL fractions separated by the U-P method were analyzed further by LC-MS/MS.

In the present study, analysis of the enzymatic fragment by LC-MS/MS was obviously an important way of detecting canine apo A-I and apo B-100. In the HDL fraction, apo A-I was detected. This result shows that the HDL fraction contained only lipoprotein having apo A-I. In the LDL fraction, both apo B-100 and apo A-I were detected. This result shows that the LDL fraction contains lipoproteins having apo A-I and apo B-100. The present results agree with those

1474



Fig. 3. SDS-PAGE analysis of the HDL+LDL (A), HDL (B) and LDL (C) fractions. The HDL+LDL, HDL and LDL fractions separated from plasma of three healthy dogs by the U-P method were analyzed using SDS-PAGE with the gradient gels. Lanes 1, 2 and 3, respectively, indicate dogs 1, 2 and 3. Proteins with estimated molecular weights of approximately 25 (a), 35 (b) and 60 kDa (c) were detected in both the HDL and the LDL fractions. The protein with a molecular weight of more than 250 kDa (d) was detected only in the LDL fraction. M: marker.

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Accession	# Co	verage	#Peptic	des #A	As MV	V (Da)	Description			
gi7395510	6	62.41	91	20	36 30)163	PREDICTED: similar to Apo A-I precursor [Canis familiaris]			to
В										
1	51		101		151		201		251	266
1 : 1MKAALLTLAV L 101 ETEVLRQEMS K	11 FLTGSQARH DLBEVKQKV	21 FWQQDEPQSP QPYLDDFQKK	31 WDRVKDLATV WQEEVELYRQ	41 YVDAVRDSGR KVAPLGSELR	51 DYVAQFEASA EGARQKLQEL	61 LGRQLNLKLL QEKLSPLGEE	71 DNWDSLSSTV LRDRARTHVD	81 TKLREQIGPV ALRAQLAPYS	91 TQEFWDNLEK DDLRERLAAR	

Fig. 4. The results of LC-MS/MS analysis for 25 kDa protein. (A) Sequence coverage (Coverage), molecular weight (MW [Da]) and description of the analyzed protein are shown as a table. Accession #, NCBI accession number; #peptides, number of peptides; #AAS, number of amino acids. (B) The coincident amino acid sequence between the analyzed protein and the canine apo A-I (gray highlighted sequence).

of Barrie *et al.* [2]. It is possible that a portion of the canine HDL was precipitated with the heparin manganese chloride solution. The canine HDL that was precipitated with canine LDL might include apo E, as Barrie *et al.* suggested [2]. It is known that an apo E-rich subfraction of HDL is present in canine plasma [16] and has a similar density and behavior to LDL [2], although this indication is not studied. Accord-

ingly, this canine HDL could be a different lipoprotein from true canine HDL. Since the characteristics of this canine lipoprotein remain unclear, so does its role. Furthermore, it is also possible that there would be lipoproteins having both apo B-100 and apo A-I in dogs. Quantitative analysis of lipoprotein fractions shows that human LDL particles having apo B-100 have certain amounts of apo A-I and apo E Α

Accession#	Coverage	#Peptides	#AAs	MW (Da)	Description			
			4500		PREDICTED: similar to			
gi73980597	53.18	752		508809	Apo B-100 precursor			
_					[Canis familiaris]			
В								
1 501	1001 1:	501 2001	25	i01 3001	3501 4001 4500			

1	11	21	31	41	51	61	71	81	91
1 MHHAAAPTVL	PFLDATORLG	OAEKGLLSPV	SHDDGGGAPR	WGFLTMWTMW	TMUTMUALCS	AGPGTGPGPP	ASAPLLPPGG	RACRPRPOAR	GAIRAREDEV
101 PESASPGCAK	GATRFKHLRK	YVYNYEAESS	SSIPGTTDSR	SATRINCKVE	LEVPOLCNFI	VRTSOCTLKE	GNGFNPKGRP	SLHKTKNSEE	FAAAMSKYEL
201 RLAIPEGKOV	LLYPEKEEPT	HILNIKRGLI	SALLVPPETE	ADKOVLFLDT	VYGNCSSDLT	VNARKANVAT	EISIERNLEK	CDHFKPISTG	VSPLALIKGM
301 VRPLSTLISS	NOSCOYTLDP	KKKHVSEAIC	KEOHLFLPFS	YENKYGIMTO	VTOTLKLEDT	PKINSRFFDE	GAREVGLAFE	STRSTSPPRO	AEAIVKTIGE
401 LQTLHVSEQN	AQRANLFNKL	VTELRGLNNE	ALTTLLPKLM	EVSSPITLQA	LIQCGQSQCY	THILQWLRSE	KANPLLIDVV	TYLVALTPEP	SAERLQEIFS
501 TAQEQQSRAT	LYALSHVVNN	YHKANPVVTQ	DLLNIADYLL	EQLODSCIGN	EDHTYLILRV	IGNMGRTMET	VAPRLTSSIL	RCIQSTEPSL	LIQKAAIQAL
601 RKMELKDEVR	EVLLQVFLDD	ASPGEKRLAA	YLMLMRSPSQ	SDINQITQLL	PREQNEQVEN	FVASHIANIL	NSEELYIKDL	KKLVEGALKG	SQLPTIMDFS
701 KFSRNYHLSK	SVSLPSLDPI	SCHIEGNLIF	DPNNYLPKES	MLKTTLTVFG	LAPADLFEIG	LEGKGFEPTL	EALFGKQGFF	PDSVNKALYW	VDDQVPDHVS
801 KVLVDHFGYT	KDGKREQDMV	NGIMLNVERL	IKDLKSKEFP	EARAYLHILG	EELGFVRLHD	LQLLGKMLLN	GVHTLAGIPQ	MIGEAIRKGL	KNDLFLHYIF
901 MDNAFELPTG	LGLQLQVSSS	GVITPGMKAG	MKVEMANLLT	ELVARPSVSM	EFVINMGIII	PDFSKTGIQM	NTNFFHETGL	EMQIAFRAGR	LKFVIPSPTR
1001 PVKLFSGSNT	LHLVSTTKTE	EVAPLNESRK	SWSTCKPLFT	GLDYCSSGAY	STANSPESAS	YYPLSRDTRF	ELELKPTGEV	EQYSASVSYE	LQREGGASVD
1101 TLTFITQTEG	LKQTEAAMMF	KYNRQSTTLS	SEIQIPDFDV	DLGTILRVSD	ESAMEKKSYR	LTLDIQNKKI	TEVILIGHVS	YDKREEGKIK	GVISIPRLQA
1201 EARSEILTQW	SPTKLLVHMD	SSATAYGLTI	SKKLGWRYDE	EKIEFEWNTG	TNVD TKKAAS	NFPMDFSDYP	KSLHMYANSL	LDHRVPQTDM	TFRHVGSKLM
1301 VATD TWLQKA	SRSLPYAQNL	QDHLSGLKEL	YLQKMGLPDF	HVPENIFLKS	DGRVKYTLNK	NSVKIEIPLP	FGGKSSKELK	MSETIQTPDL	NFKSIGFYLP
1401 SREFQVPTFT	IPKLYQLQVP	LLGVLDLSAN	IYNNLYNWSA	SYTGGNTTNN	HWSLQAQYHM	KADSVVDLLS	YSVQGSGETT	YDHRNTFTLS	CDGYLHHKLL
1501 DSNMKFSHVE	KIRNNPASKS	VLTFNTSTAW	GPQMSASLHL	DSKKKHHLYV	KEAKIDGQFT	VSSFYAKGIY	DLSYQRDSTT	GQLRGESNLR	FNSSYLQGTN
1601 QITGRYEDGT	VSLTSTSDLQ	DGIIKNTAFL	RYENYELTLK	SDISGKHEDF	ATYNKVDMTF	SKQNALVRSE	YQADYKSLRL	FILLSGSLNS	HGLELNADVL
1701 GTDRINSGAH	KATLRIAQDG	MSTSATTNLK	YNPLVLENEL	NAALGLSGAS	VRLTINGRFR	EHNAKFSLDG	KATLTEVSLG	SAYQAMILGF	DSKNVFNFKI
1801 SREGLKLSND	MMGSYAEMKL	DYTNNLNIAG	LSLDFSSKLD	NIYSSDKFYK	QNFNVQLQPY	SLVTTLSNDL	QFNALDLTNS	GKLRLEPLKL	NVGGNIKGVY
1901 QNNEIKHIYT	ISYADLSASY	KTDTVAKVQG	TEFSHRLNTN	IAGLASAIDI	STSYNSDSLH	FSNIVHSAMA	PFTMTIDALT	NSNGKLSFWG	EHTGQLYSKF
2001 LLKAEPLALT	FSHDYKGSTS	HHLPLKSSIT	TGLDHKVSAL	LTPAEQTGTW	KVKTQLNENE	YSQDFDAYND	KDKVGVELRG	RALAEPTMID	FSIPVPFLLS
2101 EYINIIDVLG	MRDAVAQPQE	FSVVASVKYD	KNQNVHTINL	PFFESLPEYF	EEKRMVIITA	LETIQRELKL	INIDQFVKKY	RTALDKLPQQ	TYGYLNSLNW
2201 ERQVSSAKEK	LITLIENYRI	TENDVQIALN	NTKISLNDKL	SQLKTYVIQF	DQYIKDNYDL	RDFKIAVAKI	IDQIIEKTKI	${\tt LD EHYHIHVN}$	LVKTIHDVCL
2301 FIEKTDFNRI	GNSTAFWIQN	VDTKYQIKIQ	IQEKLQQLKT	HIQNIDIQHL	ADQLKQQVEA	LDVRVLLDKL	RITIPFQRIK	EIIEHVRNYV	VNLMRD FEVA
2401 EKINMFGTMS	HKLIKMYGID	RHIQVLMDKL	VQLAHQYKLK	ETVQKLSSVL	QQVKIKDHFE	KLVKCIDDAI	KQLKAFSFKK	IIEGVNRFLD	MLVKKLRSFD
2501 YHQFVDEANN	KIHEVTQRLN	GEIQALELPQ	KAKALRLFIE	DIKATVSVHL	ENLKGTQISL	FFDWLQEPLN	STSLTHIKEK	FQETLEDIRE	RVYQMDIQRE
2601 VKRYLSLVGQ	VYSTLVTYIS	DWWSLAAKNL	TDFAEQYSIQ	YWAENLKALV	EQGFTVPEIQ	TTFGTMPAFE	VSLRALQEAT	FQTPDFIVPL	TDLKVPSVQI
2701NFKKLKDIEI	PPKFTTPEFT	ILNTFHVPSF	TIDLVELKAK	IIRTIDQMLS	SELQWPVPEM	YLRDLEGVDT	ILASITLPDF	CLPEITIPEF	QLPRMPHTVE
2801 VPTFGKLHSI	LKIQSPLFTL	DANADIQNVT	TSGNKAEIAA	SITARGESKL	EVLSFDFQAN	AQLSDPKISP	LVMKQSVRFS	SKYLKTEHES	EVLFFGNAIE
2901 GKSNTVAGLH	TERNTLELSN	GVLVTINNQL	TLDSSTRYFH	KLNIPKLDFS	SQAELRNEIK	TLLESGHITW	TSSGLGSWKW	ACNTSSDEGA	HESQUSFIMK
3001 EAMASFGLSN	KISSKHLKLS	QNLVFESSFP	DFSKFEIQSQ	VESQHVGHSV	LAAKGTALLG	ERKAEITGNH	NAHLNGKIIG	TLENSLFFTA	QPFELTASTN
3101 NEGNLKVSFP	LKLIGKVDFL	NNYILLESPS	TQQAGWQASA	RFNQYKYHHN	FSAGNNENSV	EAHIGINGEA	NLDFLNIPLT	IPEMTLPYTT	LTTPHVKDFS
3201 LWEKTGLKEF	LKTTRQSFDL	SVKSQYKKNK	DKHIIPIHFY	MEDFQVLSTP	GDIFIPAMGN	ITYDFSFKSG	LITLNTNVGL	YNQSDIVAHF	LTSSSSVIDG
3301 LQYKLEGTSS	LTRKRGLKLA	TALSLSNKFV	EGNHDSTISL	TKKNMEASVT	TSAKVQIPIL	RMNFKQELNG	NTRSEPTVSS	SIELIYDLNS	PKLYSTATGR
3401 VNHKLSLESL	TSYFSIESST	KGDVKGSVLS	REYSGSIAGE	ASTYLNSKST	RTSVKLQGAS	RVDGIWNLDV	KENFAGETTL	QRIYAIWEHN	MKNSLQLEDL
3501 FSSSGEHTSK	ATLELSPWRM	SALVRVHASQ	PNSFLEINYL	DQEMSLNANT	ENQKVSWKSE	VQVHSEFFQN	NIQLFNDQEE	ARLDVGSSLK	VSLWFLKDII
3601 LPIYDKSLWD	LLKLDVTTSI	DKRQYLHAAT	ALVYTENPEG	YHFSVPVQEL	ADKFIIPRLK	LSDLNSSFVI	PTFQVPFTDL	QVPSYTIDFS	BIKLYKKLST
3701 LPFSLNIPML	PRVKFPKIDV	LTRYSEPEDS	SVPFFEIIVP	ASQLTVSQFT	LLQSSSGDSA	VEYLNEVASK	FADFELPAIT	IPEQTIEIPS	IKFSVPAGIF
3801 IPSFGALTAH	FGVVSPLYNA	TWSAGLKNKV	EYVETFLNST	CSSTIQFLEY	DLNVVGTHKI	EDGMLVCRTK	GIFAHRDISA	EYKEDNRYRG	LQDWKKEIRL
3901DITSPTFTDA	HTHAOTNKNA	LSYLVASPAV	GTVAFDMEDN	NRTLKLNLYY	RPQSSLDKKL	NIFKTEWRYR	ESDEKNQIKV	SWEEEAVSKL	LISLKDNVPK
4001 ATGALYDYAN	KYHQEHTGLD	LTDASSKLRR	SLQNSADWAY	HSVLEKIDKA	DLGFQSIAKG	TIRTYQQWKD	KAQDLYQEML	LAHRDQGDFQ	GLQKDMFDRL
4101 MGVTQEYKVT	VSHQIDSLID	FLKFTKFQLP	GKPGTYTKDE	LCTMVMKEVG	QVLSQVYSKI	YSGFEILFSY	FQDLMEKSEL	IKDLEIKIPF	DSKSYKLIDV
4201 ILEFGRVLKS	LSQKPQDAFN	NLQSINTTEM	LSDLQRYLQR	AFQKIEEEIQ	RLKEKKFSYL	INHIGHKINI	IFNKYIPYVF	EFLKRNLYLN	FGRFNELVQN
4301QLQEASQQLQ	ÜLHÖIIKD LK	KEYVDSNVVS	WILRYYELEE	RIISLIKNLV	DVLKDFHSKY	TVSTADLVSQ	LSSKVEQFVQ	KDIQEYLSIL	IDADGKGKEK
4401 IAELSTSTQE	IIKSWANAMK	EIISDYHQQF	RYKLQDFLDQ	LSDYYEKFIT	ESKRLIDLSI	QNYHMFLRYI	TELLKELQSA	TVNNMIPYIK	VSPGEFTITL

Fig. 5. The results of LC-MS/MS analysis for 250 kDa protein. (A) Sequence coverage (Coverage), molecular weight (MW [Da]) and description of the analyzed protein are shown as a table. Accession#, NCBI accession number; #peptides, number of peptides; #AAS, number of amino acids. (B) The coincident amino acid sequence between the analyzed protein and the apo B-100 (gray highlighted sequence).

[5, 11]. Similarly, certain canine particles might have both apo B-100 and apo A-I, although this speculation has yet to be validated. Based on the present results, it is difficult to suggest the U-P method as an accurate method for analysis of canine lipoprotein. The U-P method cannot be a gold standard method for analyzing canine lipoprotein. Further analysis of canine lipoproteins and canine apo E is necessary. LC-MS/MS might be an efficient method for detecting

1476

canine apo E. However, analysis by SDS-PAGE showed that dogs 1–3 had different protein amounts in the HDL+LDL, HDL and LDL fractions, indicating that there are individual differences among these dogs.

Because the HDL-C and LDL-C concentrations determined by the U-P method correlated well with those determined by GP-HPLC, this method may be able to roughly separate canine HDL and LDL, meaning that the U-P method could be useful for detecting alterations in the balance of canine lipoproteins and for clarifying the clinical significance of canine lipoprotein profiles. However, the LDL/HDL ratio determined by the U-P method was quite different from that determined by GP-HPLC. Since it is possible that GP-HPLC was unable to accurately separate HDL from LDL, it is unclear which of the 2 methods is more accurate at separating canine HDL from LDL.

In conclusion, the present study indicated that the U-P method would be hard to use as a gold standard method for analysis of canine lipoprotein, because it precipitates a certain lipoprotein, which includes apo A-I, with canine LDL.

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REFERENCES

- Abate, O., Zanatta, R., Malisano, T. and Dotta, U. 2000. Canine serum protein patterns using high-resolution electrophoresis (HRE). *Vet. J.* 159: 154–160. [Medline] [CrossRef]
- Barrie, J., Nash, A. S. and Watson, T. D. G. 1993. Quantitative analysis of canine plasma lipoproteins. *J. Small Anim. Pract.* 34: 226–231. [CrossRef]
- Barrie, J., Watson, T. D. G., Stear, M. J. and Nash, A. S. 1993. Plasma cholesterol and lipoprotein concentrations in the dog: The effects of age, breed, gender and endocrine disease. *J. Small Anim. Pract.* 34: 507–512. [CrossRef]
- Barter, P., Gotto, A. M., LaRosa, J. C., Maroni, J., Szarek, M., Grundy, S. M., Kastelein, J. J., Bittner, V. and Fruchart, J. C. 2007. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. *N. Engl. J. Med.* 357: 1301–1310. [Medline] [CrossRef]
- Collins, L. A. and Olivier, M. 2010. Quantitative comparison of lipoprotein fractions derived from human plasma and serum by liquid chromatography-tandem mass spectrometry. *Proteome Sci.* 8: 42. [Medline] [CrossRef]
- Goldenberg, I., Benderly, M., Sidi, R., Boyko, V., Tenenbaum, A., Tanne, D. and Behar, S. 2009. Relation of clinical benefit of raising high-density lipoprotein cholesterol to serum levels of low-density lipoprotein cholesterol in patients with coronary heart disease (from the Bezafibrate Infarction Prevention Trial). *Am. J. Cardiol.* **103**: 41–45. [Medline] [CrossRef]
- Gordon, T., Castelli, W. P., Hjortland, M. C., Kannel, W. B. and Dawber, T. R. 1977. High density lipoprotein as a protective fac-

tor against coronary heart disease. The Framingham Study. Am. J. Med. 62: 707–714. [Medline] [CrossRef]

- Hatch, F. T. 1968. Practical methods for plasma lipoprotein analysis. Adv. Lipid Res. 6: 1–68. [Medline]
- Havel, R. J., Eder, H. A. and Bragdon, J. H. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34: 1345–1353. [Medline] [CrossRef]
- Jericó, M. M., De Camargo Chiquito, F., Kajihara, K., Moreira, M. A., Gonzales, R., Machado, F. L., Nunes, V. S., Catanozi, S. and Nakandakare, E. R. 2009. Chromatographic analysis of lipid fractions in healthy dogs and dogs with obesity or hyperadrenocorticism. *J. Vet. Diagn. Invest.* **21**: 203–207. [Medline] [CrossRef]
- Karlsson, H., Leanderson, P., Tagesson, C. and Lindahl, M. 2005. Lipoproteomics I: mapping of proteins in low-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry. *Proteomics* 5: 551–565. [Medline] [CrossRef]
- Kastelein, J. J., van der Steeg, W. A., Holme, I., Gaffney, M., Cater, N. B., Barter, P., Deedwania, P., Olsson, A. G., Boekholdt, S. M., Demicco, D. A., Szarek, M., LaRosa, J. C., Pedersen, T. R. and Grundy, S. M. 2008. Lipids, apolipoproteins, and their ratios in relation to cardiovascular events with statin treatment. *Circulation* 117: 3002–3009. [Medline] [CrossRef]
- Krauss, R. M. and Burke, D. J. 1982. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. *J. lipid Res.* 23: 97–104. [Medline]
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685. [Medline] [CrossRef]
- Mahley, R. W. and Weisgraber, K. H. 1974. Canine lipoproteins and atherosclerosis. I. Isolation and characterization of plasma lipoprotein from control dogs. *Circ. Res.* 35: 713–721. [Medline] [CrossRef]
- Mahley, R. W. and Innerarity, T. L. 1977. Interaction of canine and swine lipoproteins with low density lipoprotein receptor of fibroblasts as correlated with heparin/manganese precipitability. *J. Biol. Chem.* 252: 3980–3986. [Medline]
- Mizutani, H., Sako, T., Arai, N., Kuriyama, K., Yoshimura, I., Mori, A., Iwase, K. and Hirose, H. 2010. Application of gel permeation HPLC for lipoprotein profiling in dogs. *J. Vet. Med. Sci.* 72: 813–817. [Medline] [CrossRef]
- Puppione, D. L., Bassilian, S., Souda, P., MacDonald, M. H., Halgand, F. and Whitelegge, J. P. 2008. Mass spectral analysis of the apolipoproteins on dog (Canis lupus familiaris) high density lipoproteins. Detection of apolipoprotein A-II. *Comp. Biochem. Physiol. Part D Genomics Proteomics* 3: 290–296. [Medline] [CrossRef]
- Rosenfeld, J., Capdevielle, J., Guillemot, J. C. and Ferrara, P. 1992. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal. Biochem.* 203: 173–179. [Medline] [CrossRef]
- Usui, S., Hara, Y., Hosaki, S. and Okazaki, M. 2002. A new online dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC. *J. Lipid Res.* 43: 805–814. [Medline]
- Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T. and Mann, M. 1996. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* 379: 466–469. [Medline] [CrossRef]