

## Quantification of horse plasma proteins altered by xylazine using the fluorogenic derivatization-liquid chromatography-tandem mass spectrometry

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*In the doping tests currently used in horse racing, prohibited substances or their metabolites are usually directly detected in urine or blood samples. However, despite their lasting pharmaceutical effects, some prohibited substances are rapidly eliminated from horse urine and blood, making them difficult to detect. Therefore, new indirect biomarkers for doping, such as plasma proteins that are increased by the prohibited substances, have recently attracted much attention. Here, a fluorogenic derivatization-liquid chromatography-tandem mass spectrometry (FD-LC-MS/MS) method was adopted for horse plasma proteomics analysis, in order to identify plasma proteins whose concentrations were altered in response to xylazine in Thoroughbred horses. Xylazine, which is rapidly absorbed and eliminated and has possibility of the change in the levels of plasma proteins, was selected as a model drug. Of the ten plasma proteins identified, four proteins, including three acute phase proteins (haptoglobin, ceruloplasmin, and  $\alpha$ -2-macroglobulin-like), were significantly increased after xylazine administration. Therefore, our present approach might be useful in identifying indirect biomarkers of drug administration.*

**Key words:** doping, FD-LC-MS/MS, plasma proteomics, xylazine

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Doping and inappropriate use of medicines not only threaten the integrity and reputation of horse racing but also are highly detrimental to fair competition and the welfare of race horses [23]. The doping tests generally used in horse racing directly detect prohibited substances, including legitimate drugs or their metabolites, in urine or blood samples, providing evidence that the substances were used [23]. However, despite their lasting pharmaceutical effects, some prohibited substances, such as anabolic steroids and peptides (insulin-like growth factor-1 etc.), are difficult to

detect due to their rapid elimination from blood and urine. Therefore, in recent years, to maintain the health of horses and fair competition, other evidence of rapidly eliminated banned substances, such as changes in plasma protein levels, has been actively pursued [5]. Proteins perform cellular various functions as either direct or indirect bio-functional molecules, and changes in the abundance of proteins are principally a reflection of alterations in biological processes or states. To identify indirect biomarkers of doping, Barton *et al.* investigated the changes in protein levels after administration of testosterone, which is a long-acting drug, using a high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS) method. They demonstrated that, among the 49 proteins detected in horse plasma, the expressions of two proteins, clusterin and leucine-rich alpha-2-glycoprotein, were induced by testosterone [5].

Recently, we developed a novel proteomics technique, fluorogenic derivatization-liquid chromatography-tandem

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mass spectrometry (FD-LC-MS/MS). The method includes fluorogenic derivatization (FD) of proteins with fluorogenic reagents, such as 7-chloro-N-[2-(dimethylamino) ethyl]-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-CI), HPLC separation of the proteins, and detection/quantification of the derivatized proteins using with HPLC-fluorometry. Specifically, the target protein is isolated by HPLC-fluorometry, digested with trypsin, and then identified by nano-LC/MS/MS. We previously used this method for proteomics analysis of various biological samples, including cancer cell lines, mouse liver and brain, and horse muscle [3, 11, 12, 15, 16], to identify biomarkers that reflect key biological processes or states. The method is relatively simple, sensitive, and reproducible, and allows for the quantification and identification of proteins in a biological sample, which might be applicable to investigating the changes in protein levels after administration of drugs to horses.

In this study, we used the FD-LC-MS/MS method to identify the altered plasma proteins (biomarkers) in Thoroughbred horse plasma induced by a short-acting legitimate drug, xylazine [10, 21]. Since xylazine, which is an agonist of  $\alpha_2$ -adrenergic receptors, is rapidly absorbed and eliminated (half-life: ca. 30–50 min), and is often used for sedation, anesthesia, and analgesia in horses [20], we are interested in its effect on plasma proteins instead of long-acting doping agent, testosterone, studied by Barton *et al.* [5]. The other reason is that xylazine was previously reported to cause elevated levels of TNF after acute lung injury and pulmonary edema in rats [2], and it induces severe pulmonary parenchymal damage when administered at clinical sedative doses in sheep [7]. Therefore, plasma protein levels might be changed when xylazine is administered to horses.

All experimental procedures were approved by the Animal Welfare and Ethics Committee of Nippon Veterinary and Life Science University. Three Thoroughbred gelding horses, ranging in age from 12 to 21 years and weighing between 480 and 500 kg, were used in this study. All horses were dosed intravenously with 1.0 mg/kg of xylazine hydrochloride and housed at Nippon Veterinary and Life Science University.

Blood samples were collected before xylazine administration and at 3, 48, and 120 hr after administration. Blood from one horse was not sampled at 120 hr due to his unruliness. Samples were centrifuged at  $1,000 \times g$  for 10 min at  $4.0^\circ\text{C}$ , and the plasma obtained was stored at  $-80^\circ\text{C}$  until use.

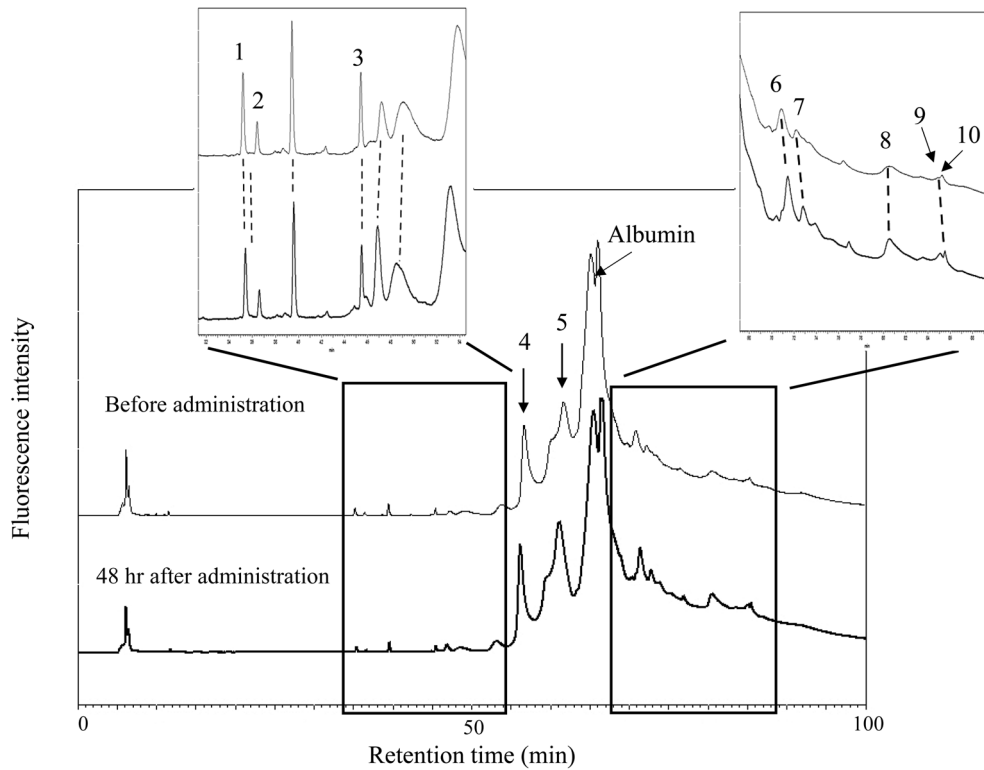
Since excess albumin interferes with proteomics analysis [6], excess albumin was removed from the plasma using a BioMag<sup>®</sup> ProMax Albumin Removal Kit (Polysciences, Warrington, PA, U.S.A.). The FD-LC-MS/MS method consists of two parts, quantification of the fluorogenic-

derivatized proteins and identification of peptides derived from tryptic digestion of each derivatized protein. First, the eluted sample was subjected to fluorogenic derivatization with DAABD-CI and then injected into an HPLC-fluorometric detector in order to quantify each protein in the sample. The conditions for the FD reaction and HPLC-fluorometric system were as previously reported [14], except for the injection volume (50  $\mu\text{l}$  of the FD reaction mixture) and the use of a Phenomenex Aeris WIDEPORE 3.6  $\mu\text{m}$  C4 column (250  $\times$  4.6 mm i.d.; Phenomenex, Torrance, CA, U.S.A.). Next, each protein peak was isolated after it passed through the detector and was then digested with trypsin. Finally, each peptide mixture derived from a single peak of the derivatized protein was injected into a nano-LC/MS/MS system. To identify each protein, the raw MS/MS data were matched to a peptide sequence database using the Mascot software. The conditions for the nano-LC/MS/MS were as previously reported [14]. The database used was the *Equus Caballus* database of the National Center for Biotechnology Information (NCBI; 76,330 sequences, 31,906,455 residues).

The quantification accuracy of the method was determined based on the reproducibility of the peak area, and the standard error of the mean (SEM, %) was determined for each between-day peak.

To identify potential biomarkers of xylazine administration, the peak areas in the HPLC-fluorometric chromatograms obtained before xylazine administration and at 3, 48, and 120 hr after administration were compared. The differences in protein expression levels were evaluated using the Student's *t*-test.

Figure 1 shows typical HPLC-fluorometric chromatograms of horse plasma obtained before and 48 hr after xylazine administration. After quantification of protein peaks (peaks 1–10 in Fig. 1), each peak was identified as described above (Table 1). The identified proteins were as follows: 60-kDa heat shock protein, mitochondrial; immunoglobulin lambda light chain constant region, partial; haptoglobin;  $\beta$ -2-glycoprotein 1; hemopexin; complement factor B; ceruloplasmin; immunoglobulin gamma 4 heavy chain, partial; and two  $\alpha$ -2-macroglobulin-like proteins. Albumin, which was not removed completely by the pretreatment and eluted at around 65 min, was excluded from the subsequent differential analysis. The peaks 9 and 10 were identified as  $\alpha$ -2-macroglobulin-like protein. It is possible that the primary structure of one or both of these proteins was changed by isomerization or posttranslational modification, causing two peaks of the  $\alpha$ -2-macroglobulin-like protein to appear. However, the primary structure of the  $\alpha$ -2-macroglobulin-like protein should be determined in a future experiment. Among the ten proteins identified, four proteins, haptoglobin,  $\beta$ -2-glycoprotein 1, ceruloplasmin,



**Fig. 1.** Chromatograms of proteins in xylazine-administered horse plasma derivatized with DAABD-Cl. The chromatograms shown at the top and bottom were obtained from horse plasma (containing  $5.6 \mu\text{g}$  of protein) before xylazine administration and 48 hr after administration, respectively. The elution conditions are described in the Materials and Methods. The fluorescence of DAABD (ex. 395 nm, em. 505 nm) was detected.

and  $\alpha$ -2-macroglobulin-like protein, significantly increased after administration of xylazine, as shown in Fig. 2. We found that the concentration of haptoglobin increased at 3 hr after administration ( $1.24 \pm 0.44$ -fold), significantly increased at 48 hr after administration ( $2.23 \pm 0.42$ -fold), and then decreased at 120 hr after administration ( $1.30 \pm 0.05$ -fold; Fig. 2A). The concentration of  $\beta$ -2-glycoprotein 1, which prevents activation of the intrinsic blood coagulation cascade by binding to phospholipids on the surface of damaged cells [1, 9], increased slightly at 3 hr after administration ( $1.04 \pm 0.16$ -fold), significantly increased at 48 hr after administration ( $1.23 \pm 0.06$ -fold), and then decreased at 120 hr after administration ( $1.02 \pm 0.25$ -fold; Fig. 2B). Ceruloplasmin significantly increased and reached its maximum at 3 hr after administration ( $1.72 \pm 0.22$ -fold), and then gradually decreased (48 hr,  $1.54 \pm 0.33$ -fold; 120 hr,  $1.16 \pm 0.61$ -fold; Fig. 2C). The fast-eluting  $\alpha$ -2-macroglobulin-like protein increased at 3 hr after administration ( $2.72 \pm 0.26$ -fold) and further increased at 48 hr after administration ( $10.70 \pm 4.27$ -fold); then, its concentration decreased at 120 hr after administration ( $3.83$

$\pm 3.18$ -fold; Fig. 2D).

The three proteins (haptoglobin, ceruloplasmin, and  $\alpha$ -2-macroglobulin-like protein) are categorized as acute phase proteins [8, 17, 19]. Acute phase proteins are defined as proteins whose serum concentrations change in response to the action of inflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor (TNF)- $\alpha$ . The acute phase response is part of the innate immune response, and acute phase proteins mediate such systemic effects as fever, leukocytosis, increased cortisol levels, decreased thyroxine levels, and decreased serum iron, as well as many others [22]. Xylazine reportedly elevated the levels of TNF after acute lung injury and pulmonary edema in rats [2]. Celly *et al.* [7] reported that xylazine induces severe pulmonary parenchymal damage when administered at clinical sedative doses in sheep. Furthermore, Atwal *et al.* [4] reported the unique shape of macrophages in horse lungs and found that halothane, an anesthetic, caused structural changes in horse lungs. Since it is plausible that xylazine possesses characteristics similar to those of halothane, it is possible that the elevated TNF levels induced by the administration

**Table 1.** Identified proteins and their concentration ratios in xylazine-administered horse plasma derivatized with DAABD-Cl

Peak number <sup>a</sup>	Protein name	Ratio $\pm$ SEM <sup>b</sup>			Accession number <sup>c</sup>	Score <sup>d</sup>	Sequence coverage (%) <sup>e</sup>
		3 hr/pre (n=3)	48 hr/pre	120 hr/pre (n=2)			
1	60 kDa Heat shock protein, mitochondrial	1.02 $\pm$ 0.05	1.01 $\pm$ 0.14	1.06 $\pm$ 0.01	gi 149730823	126	4
2	Immunoglobulin lambda light chain constant region, partial	0.68 $\pm$ 0.23	0.71 $\pm$ 0.14	1.07 $\pm$ 0.03	gi 356494355	48	37
3	Haptoglobin	1.24 $\pm$ 0.44	2.23 $\pm$ 0.42*	1.30 $\pm$ 0.05	gi 149699777	293	6
4	$\beta$ -2-Glycoprotein 1	1.04 $\pm$ 0.16	1.23 $\pm$ 0.06**	1.02 $\pm$ 0.25	gi 149723623	315	51
5	Hemopexin	0.77 $\pm$ 0.24	1.89 $\pm$ 0.40	1.31 $\pm$ 0.28	gi 545222763	544	53
6	Complement factor B	1.58 $\pm$ 0.44	1.31 $\pm$ 0.19	1.24 $\pm$ 0.19	gi 149732066	1,242	55
7	Ceruloplasmin	1.72 $\pm$ 0.22*	1.54 $\pm$ 0.33	1.16 $\pm$ 0.61	gi 149729967	795	28
8	Immunoglobulin gamma 4 heavy chain, partial	1.00 $\pm$ 0.07	1.30 $\pm$ 0.11	1.26 $\pm$ 0.34	gi 42528293	97	22
9	$\alpha$ -2-Macroglobulin-like	2.72 $\pm$ 0.26	10.70 $\pm$ 4.27**	3.83 $\pm$ 3.18	gi 194211675	963	38
10	$\alpha$ -2-Macroglobulin-like	1.43 $\pm$ 0.24	1.51 $\pm$ 0.33	1.88 $\pm$ 0.13	gi 194211675	717	36

<sup>a</sup>Peak numbers correspond to those shown in Fig. 1. <sup>b</sup>Each concentration was divided by the concentration at 0 hr (before administration [pre]). <sup>c</sup>The accession number is a series of digits that are assigned consecutively to each sequence record processed by NCBI. <sup>d</sup>Identified protein database entries are presented in descending order in the search results, based on scores. A protein that has more matching MS/MS scans in the search receives a higher overall score and is ranked higher in the search results. The top listed match is often the most abundant protein in the sample. <sup>e</sup>Sequence coverage is the percentage of the database protein sequence covered by the matching peptides. Asterisks indicate significant differences from the peak at 0 hr (\* $P \leq 0.01$ , \*\* $P \leq 0.05$ ).

of xylazine also led to lung injury, followed by inflammatory cytokine release, resulting in increased levels of these acute phase proteins in horse plasma as a means to enhance the innate immune response. Further studies determining the TNF levels in treated plasma samples and examination for possible damage to the lungs of horses are needed.

Considering the intended application of these results as doping tests, additional studies of these four potential biomarkers of xylazine administration are needed. For example, in order to detect more proteins and prolong the appearance time of concentration changes, administration of 42 mg/kg (*im* injection) as performed by Amouzadeh *et al.* [2] should be investigated, as the present experiment administered 1.0 mg/kg (*iv* injection). A significant increase of the four proteins might be a possible proof of xylazine administration. However, acute phase proteins are known to be induced by many causes with physiological phenomena. Therefore, to detect other biomarkers except for the four proteins might be required.

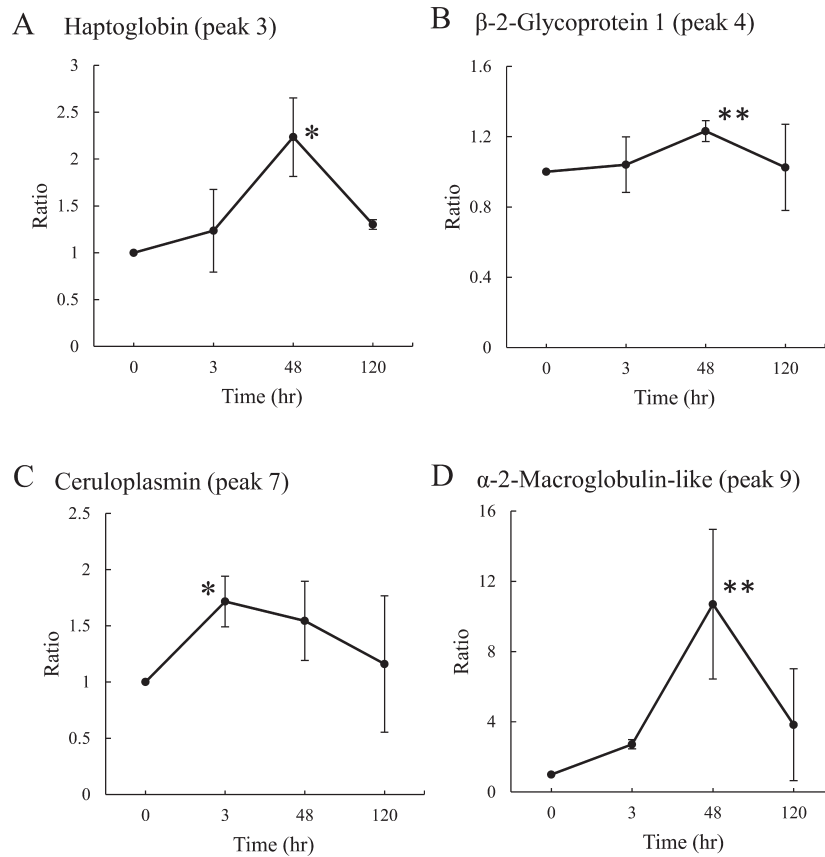
Before using FD-LC-MS/MS for our horse plasma proteomics analysis, we optimized the experimental conditions. It is necessary to remove an abundant protein, albumin, in plasma for proteomics analysis. Previously, we tested commercially available immunoaffinity columns that are normally used in proteomics studies for removing albumin from plasma [13], and we encountered with a problem, nonselective protein loss. This loss was an impediment to quantitative analyses using not only FD-LC-MS/MS but also other proteomics methods. The albumin removal kit we used in the present study seemed to be appropriate in

terms of lower nonselective loss compared to the immunoaffinity columns. However, the number of plasma proteins detected was fewer than we expected, probably because of the lower ability of the albumin kit to capture proteins. To address this problem, we need to develop a novel plasma pretreatment approach to comprehensively analyze all plasma proteins. We also investigated the HPLC conditions. Since the conventional nonporous reversed-phase column (C18, 150 or 250  $\times$  4.6 mm i.d.) that is generally used for FD-LC-MS/MS made it difficult to effectively separate the treated plasma sample, we successfully used a core-shell particles C4 column [18], which has weaker retention than the C18 column.

In conclusion, we analyzed the proteomics changes in horse plasma after xylazine administration using the FD-LC-MS/MS method, and were able to determine some of the altered plasma proteins. Furthermore, we demonstrated for the first time that several acute phase proteins were significantly altered. Our present approach might be useful in identifying indirect biomarkers of drug administration. Further examination is required to obtain the detailed data on xylazine administration as well as other drugs administration before applying the observed results to doping testing.

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**Fig. 2.** Changes in concentration ratios of proteins significantly altered following xylazine administration. (A) Haptoglobin (peak 3), (B)  $\beta$ -2-glycoprotein 1 (peak 4), (C) ceruloplasmin (peak 7), and (D)  $\alpha$ -2-macroglobulin-like (peak 9). The peak numbers, indicated in parentheses, are the same as those shown in Fig. 1 and Table 1. Mean concentration ratio values  $\pm$  SEM (n=3 at 0, 3, and 48 hr; n=2 at 120 hr) are plotted. Asterisks indicate significant differences from the peak at 0 hr (\* $P$  $\leq$ 0.01; \*\* $P$  $\leq$ 0.05).

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