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Origin identification of recombinant glycoprotein hormones by monitoring human-type glycans

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Abstract

Previously, we have shown that CHO-expressed recombinant erythropoiesis stimulating agents (ESA) are lacking several human type glycans and do not show any interaction with SNA, SSA and TJA-I lectins. Lack of interaction with recombinant hCG, LH and FSH and strong interaction with those of human origin were also confirmed in the present study. The results indicate that recombinant glycoprotein hormones expressed in CHO cell lines do not have sialyl alpha2,6-Gal/-GalNAc moieties and show a possibility to apply the findings of the origin identification based on the presence or absence of these moieties. Lectin-carbohydrate binding was reported to be weak, however, binding of interactive lectin and the target glycoprotein with the large glycan attachments were found to be more stable than expected. The complex was kept without significant decrease even after washing for 97 hours and showed the applicability of the use of diagnostic lectins for origin specific purification for the further analyses.

Introduction

Chinese hamster ovary (CHO) cells are known to produce N-Linked sugar chains with the terminal NeuAc alpha2,3-Gal beta1,4-GluNAc-R moiety and lack Gal alpha2,6-sialyltransferase [1]. We therefore hypothesised that CHO-expressed recombinant glycoprotein hormones could be differentiated from those in man by monitoring the sialyl alpha2,6-linked Gal/GalNAc moieties. However, identification and separation of isomeric glycans by mass spectrometry is not easy and time consuming. Results of our recent lectin-glycan interaction studies confirm that CHO-expressed ESAs, hCG, LH and FSH are lacking sialyl-alpha2,6 Gal/ GalNAc and do not show any interaction with SNA, SSA and TJA-I lectins while glycoprotein hormones purified from human urine or EPO-delta expressed in HT-1080 human fibrosarcoma cell lines show a strong interaction with those lectins. WGA is a lectin that recognizes chitin oligomer, (GluNAc)n or terminal sialic acid, and currently used to separate ESAs depending on the sialic acid content or pKa [2]. Apart from the WGA approach, the aim of our study is to establish methods for separating exogenous and endogenous glycoprotein hormones using origin-specific lectin-glycan interactions, to allow high throughput detection of exogenous glycoprotein hormones.

Experimental

Ovitrelle (hCG), Gonalef (FSH), ESPO Injection (Epoetin-alpha), EPOGIN Injection Syringe (Epoetin-beta), Epoetin Alfa BS Injection Syringe (Epoetin-kappa), NESP Injection Plastic Syringe (Darbepoetin: DPO) and MIRCERA Injection Syringe (CERA) were from commercial sources. Binocrit (Epoetin-alpha) was a kind gift from Sandoz. Follistim (FSH), Gonapure (FSH), Folyrmon (FSH) and Gonatropin (HCG), Human Chorionic Gonadotrophin, HCG Mochida (HCG), Gestron (HCG) and Pregnyl (HCG) were of human origin and obtained from commercial sources. Dynepo (Epoetin-delta) was kindly gifted by the WADA accredited London laboratory. Proteomic grade trypsin and recombinant N-glycosidase F were purchased from Sigma-Aldrich and Roche Applied Science, respectively. The active ingredient of these pharmaceutical products was assayed by sandwich EIA on a Tosoh AIA-600II and Immulite 1000. HCG and beta-hCG kits for AIA-600II and free beta-hCGII kit for Immulite 1000 were used to measure intact hCG, intact + free-beta-hCG and free-beta-hCG in accordance with the manufacturers' instructions. The sequence of the glycan-detached tryptic peptides was analyzed by LC/ESI-QqLIT-MS with the results verified by LC/ESI-IT-TOFMS according to our published standard protocol [3].

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After PNGase F digestion of tryptic digests, the cleaved glycans were captured and washed on hydrazine beads [4]. Profiles of the labeled glycans were monitored by MALDI-IT-TOFMS instrument AXIMA resonance from Shimadzu. Cooling gas was He, and ion abundance was measured in raster scan mode for 10,000 counts each at 1,000 points per MALDI well. A N_2 Laser at 337.1 nm was used. Matrix was 10 mg/mL recrystallized 2,5-dihydroxy benzoic acid in 30%-acetonitrile [3]. Lectin-glycan interaction was monitored either by single reaction or by lectin microarray detection which covers 45 kinds of triplicated lectins spots using Glycostation® from Glycotechnica [5]. 20 µL of approximately 50 µg/mL glycan solution that corresponds to about 1 µg is mixed with 100 µg of Cy3 mono-reactive dye, and the mixture was reacted in the dark at room temperature for 1 hour. After removal of excess Cy3 dye by gel filtration, the reaction mixture was diluted to about 4 µg/mL with probing solution, then the analyte was further diluted 5 to 1,000 times with probing solution. Before sample loading, each well of the lectin microarray chip (LecChip®) was regenerated by washing 3 times with probing solution. 100 µL of analyte that corresponds to sub pg to pg absolute was loaded in each well. The Lectin-glycan complex was formed by incubation in a humidifying boxin the dark at 4 °C overnight.
For fractionation of glycoproteins, immobilized SSA or SNA lectin on agarose gel was used for affinity column chromatography as a model. 2 mL of SSA agarose gel was washed with 4 mL x 5 of PBS, and the gel in the cartridge was well incubated with 1 mL of sample solution at room temperature for 60 minutes. About 1 mL of the first elute was collected in a 2 mL test tube, and the second fraction was collected in a 10 mL test tube by loading 5 mL x 2 of PBS buffer onto the column. The remaining glycans on the agarose gel were then eluted with 5 mL x 2 of 0.2 M lactose in PBS, and 10 mL of the third fraction was collected.

Results and Discussion

Glycan binding site analysis:
Results of MRM analysis by LC/ESI-Qq-LIT-MS showed that the glycan detached tryptic peptides EAENITTGCAEHC-SLNENITVPDTK (m/z:935.7>460.2) and GQALLVNSSQPWEPLQLHVDK (m/z:787.4>949.5) were common for Epoetins and CERA. In DPO, those fragments were replaced by EAENITTGCNEHCSLNENITVPDTK (m/z:938.4>460.2) and GQALLVN-SSQVNETLQLHVDK (m/z:765.7>498.3), respectively, and the results demonstrated the presence of two additional N-glycans at Asn 24 and Asn 83 of the core peptide. A short tryptic peptide VNFYAWK (m/z: 464.2>714.4) was common for Epoetins and CERA. However, the abundance of this peptide is significantly suppressed in CERA; indicating pegylation at Lys 52.
Monitoring N-glycan binding site was useful to classify generation of ESAs [3] and for identification of biosimilars, however, no information to differentiate human EPO and Epoetin could be obtained. N-glycan composition of original epoetins and the biosimilars were not the same, and monitoring of the difference was considered to be useful for the evaluation of chemo-equivalence of biosimilars (data not shown).
In Table 1, sequence, ion abundances and percentage of O-glycan on Ser 126 of ESAs calculated from triplicate LC/ESI-IT-TOFMS analyses are shown. The O-glycan of ESAs consisted of Hexose (Hex) and N-Acethyl hexose (HexNAc) with zero to two neuraminic acids (NeuAc) with one to three acetates (Ac) and the results on acetylation status of O-glycans was in good agreement with those published by Shahrokh et al. [6] and subsequently reported by Llop et al. and Reichel [7,8]. Major components for Epoetin and the biosimilar were Hex-HexNAc-NeuAc and Hex-HexNAc-$(NeuAc)_2$. Extent of acetylation of Binocrit and EPO BRP was found to be higher than those of the original epoetins, and different acetylation status of O-glycan between original epoetin and Binocrit or lot-to-lot difference within the product was suggested. As expected, O-glycan of DPO was highly acetylated and Hex-HexNAc-NeuAcAc, Hex-HexNAc-NeuAc-NeuAcAc and Hex-HexNAc-$(NeuAcAc)_2$ were predominant in addition to Hex-HexNAc-NeuAc and Hex-HexNAc-$(NeuAc)_2$.
No further study on O-glycans of ESA was performed because the major aim of our study was not to focus on ESAs only but to establish a high throughput comprehensive screening of recombinant glycoprotein hormones.

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Composition of *O*-Linked glycan				m/z	ESPO	EPOGIN	EPO-k	BINOCRIT	BRP	NESP	CERA
					Absolute abundance (Mean area n=3)						
Hex	HexNAc			915.95	24038212	32962211	15976534	12372154	28950501	18700770	50743100
Hex	HexNAc	NeuAc		1061.50	155878177	168041227	94355559	69909619	168062581	136161633	305825754
Hex	HexNAc	NeuAc	NeuAc	1207.05	72564115	86612528	48224564	53500702	80269935	83760558	120797849
Hex	HexNAc	NeuAcAc		1082.50	27036475	9433938	13932354	25959000	54212827	51225507	43718866
Hex	HexNAc	NeuAcAcAc		1103.52	13266373	1767008	10170952	19892236	40981026	31303181	15787667
Hex	HexNAc	NeuAc	NeuAcAc	1228.06	15698097	5261050	7541648	24896522	38062665	80514674	44443474
Hex	HexNAc	NeuAcAc	NeuAcAc	1249.06	7080527	2029473	5390265	19110385	30801376	49069667	14921133
Hex	HexNAc	NeuAcAc	NeuAcAcAc	1270.07	1520043	94233	911449	6200167	8072206	13874090	3740313
			Acetate		Percentage (%)						
Hex	HexNAc				7.6	10.8	8.1	5.3	6.4	4.0	8.5
Hex	HexNAc	NeuAc			49.2	54.9	48.0	30.2	37.4	29.3	51.0
Hex	HexNAc	NeuAc	NeuAc		22.9	28.3	24.5	23.1	17.9	18.0	20.1
Hex	HexNAc	NeuAcAc		1Ac	8.5	3.1	7.1	11.2	12.1	11.0	7.3
Hex	HexNAc	NeuAcAcAc		2Ac	4.2	0.6	5.2	8.6	9.1	6.7	2.6
Hex	HexNAc	NeuAc	NeuAcAc	1Ac	5.0	1.7	3.8	10.7	8.5	17.3	7.4
Hex	HexNAc	NeuAcAc	NeuAcAc	2Ac	2.2	0.7	2.7	8.2	6.9	10.6	2.5
Hex	HexNAc	NeuAcAc	NeuAcAcAc	3Ac	0.5	0.0	0.5	2.7	1.8	3.0	0.6

Table 1: Structure and composition of O-linked glycan of ESA on Serine 126
EPO BRP is a mixture of Epoetin-alpha and -beta. However, the acetylation of the O-glycan of EPO BRP was seen to a larger extent; suggesting a lot-to-lot difference. Extent of acetylation of Binocrit was found to be higher than those of original Epoetins and that of CERA was similar as original Epoetin-alpha. As expected, O-glycan of DPO was highly acetylated and Hex-HexNAc-NeuAcAc, Hex-HexNAc-NeuAc-NeuAcAc and Hex-HexNAc-$(NeuAcAc)_2$ were also predominant.

Linearity of lectin microarray: Dose response of the detector signal ranged between 4 to 4,000 ng/mL (approximately 0.01 to 10 pg absolute) when focused on a single lectin, however, sample concentration was carefully adjusted to the linearlity range. Dynamic ranges of the lectin spots vary and the range depends on the glycan composition of the analyte (Figure 1). To differentiate exogenous and endogenous glycoproteins, for example, monitoring several diagnostic signals only is necessary and the dynamic range for SSA, SNA and TJA-I is close to each other, so that a narrow range of the detector signals of the lectin microarray does not suppress the detectability of glycoprotein hormones.

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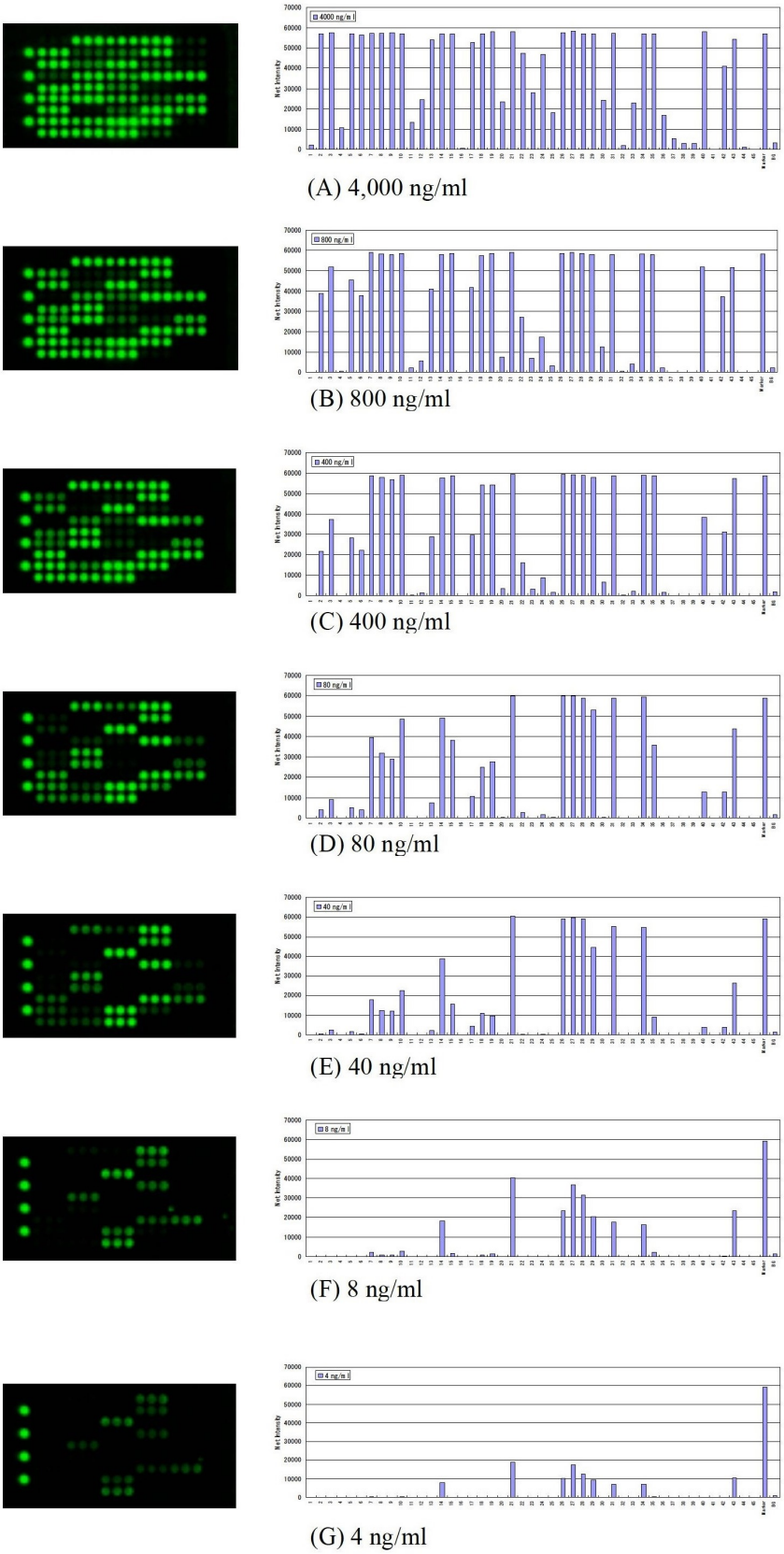

Figure 1: Linearity of lectin microarray signal. Starting with 4 to 4,000 ng/mL hCG solutions, about sub -pg to pg absolute per well of the analyte was loaded on chip for measuring the evanescent fluorescence. Dynamic range of full lectin-glycan interaction spectra was limited but higher sensitivity could be obtained when the gain of the detector signal was adjusted based on the S/N ratio of any of the single lectin spots.

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Robustness of lectin-glycan complex: In general, the affinity of lectin with the target carbohydrate is 100 times lower than that of the corresponding antibody-antigen reaction, and therefore lectin-glycan interaction is being monitored without washing step [5]. It has been confirmed by our studies that for protein with large glycan attachments, such as WADA prohibited glycoproteins, the lectin-glycan complex was very robust. For all target glycoproteins studied, the lectin-glycan complex was stable for 97 hours even after washing with a probe solution, and cleaner and lower-background signals could be obtained after washing the array chip.

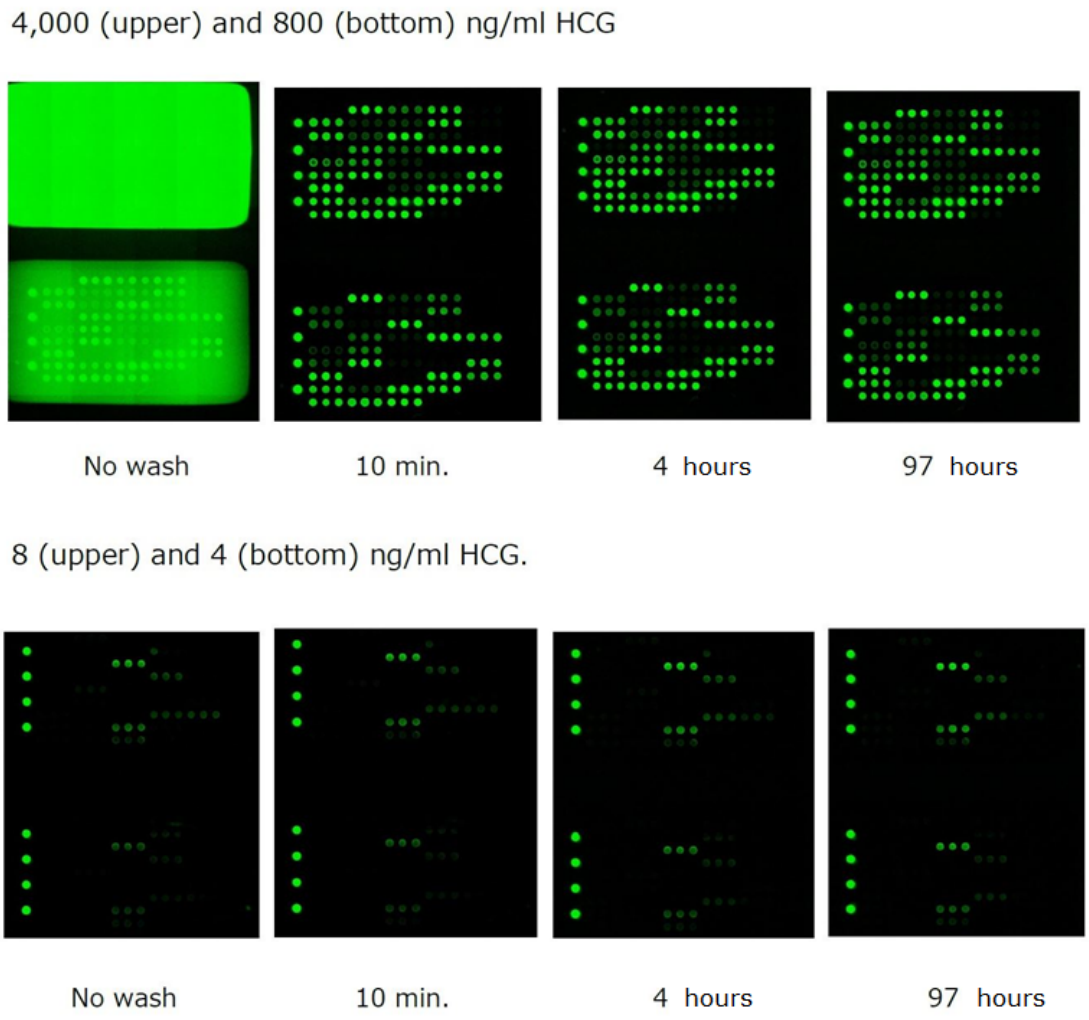

Figure 2: Robustness of lectin-glycan interaction signals
High background of the lectin-glycan interaction signal is observed when the carbohydrate content of the analyte is too high. The background could be removed and a cleaner signal can be obtained by washing the chip with a proving solution, because the lectin-glycan complex is more stable than expected when the glycoprotein has large glycan attachments.

Specificity of lectin-glycan interaction: Detector response of the lectin-glycan interaction for ESAs or for HCG from several sources is listed in Table 2 and Table 3. In Table 4, ESA with different core peptides, pegylation status or different number of glycan attachments are shown. Among those products, Epoetin, biosimilars, DPO and CERA did not show any significant interaction with the diagnostic lectins. The lack of the lectin-glycan interaction did not depend on the sequence of their core peptide. EPO-delta, Epoetin-alpha from human cell-lines, was the sole recombinant ESA that showed the interaction with diagnostic lectins. Ovitrelle is the HCG from CHO cell lines (Table 3). It did not show any interaction with three diagnostic lectins at all. Other HCGs are purified from human urine as the activeingredient of those products and has shown significant and strong lectin-glycan interactions.

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Evanescent FL	EPO-α	EPO-β	EPO-κ	NESP	EPO-BRP	CERA	Binocrit	EPO-δ
Siaα2-6Gal/GalNAc								
SNA	20.2	24.6	0.0	22.7	2.8	45.5	3.4	2184.3
SSA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1951.6
TJA-I	34.1	43.4	0.0	10.6	0.0	50.5	0.0	2417.2
OligoChitin, (GluNAc)n, Sia								
WGA	2414.9	4343.3	3056.4	7352.0	1142.9	4215.1	2001.7	5618.3
Backgroud	2176.7	475.8	545.9	486.6	443.4	1652.0	565.1	976.6
Standard Deviation								
SNA	14.6	8.7	0.0	16.1	3.9	17.3	4.8	140.1
SSA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	162.0
TJA-I	48.3	15.6	0.0	15.0	0.0	16.1	0.0	242.0
WGA	169.5	333.9	215.2	355.4	75.3	242.1	82.6	340.7
Background	205.5	38.3	47.9	64.1	52.4	230.8	31.0	46.8

Table 2: Specificity of lectin-glycan interaction for ESA
SNA, SSA and TJA-I are known to recognize sialyl alpha2-6 galactose/ N-acetyl galactosamine and essentially interact only with endogenous EPO. WGA recognizes terminal sialyc acid, N-acethyl glucosamine repeats etc., and interacts with all ESAs studied. Among three origin specific lectins, SSA was highest specific lectin to human type glycans.

	HCG Ovitrelle	HCG Gonatropin	HCG human	HCG Mochida	HCG Gestron	HCG Pregnyl	FSH Gonaref	FSH Follistim	FSH Gonapure	FSH Folyrmon
	CHO			Human Urine			CHO		Human Urine	
SNA	0.0	2506.0	4107.6	1990.4	918.1	18538.6	0.0	0.0	53575.7	58720.3
SSA	0.0	1914.2	3513.7	1726.0	883.2	18296.0	0.0	0.0	50687.3	59327.2
TJA–I	0.0	5810.8	8035.4	4394.2	2684.6	28898.2	0.0	0.0	58439.1	58756.6
WGA	20623.0	37247.1	36734.8	24369.2	23475.9	48814.7	3958.1	431.7	12079.5	18322.5
Backgroud	183.5	1054.0	1019.8	1114.5	1059.2	1352.1	1681.7	557.0	1392.0	1593.3

Table 3: Specificity of lectin-glycan interaction for HCG and FSH
As same as in case of ESAs, HCG and FSH with human origin have shown strong interaction with SNA, SSA and TJA-I. HCG and FSH that genetically manufactured by expressing the DNA in CHO cell line did not show any interaction with those three lectins. WGA has recognized all those gonadal hormones.

	Applied (mIU/ml)	Reaction time (hr)	Conc. (mIU/ml)	Recovery (DPO %)
Urine 1	173	1	187	108
Urine 2	282	1	243	86.2
Urine 3	173	66	130	75.1

Table 4: Recovery of Darbepoetin
Blank urines spiked with known ammounts of DPO was extracted using SSA-agarose gel and the recovery was estimated. After incubating the suspension for 1 to 66 hours, DPO was eluted and recoveries between 75.1 and 108% were obtained. Shorter incubation time such as for 1 hour was enough to capture endogenous EPO and to collect DPO.

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Purification of DPO as a model for origin specific extraction: As a model, known amounts of DPO were added to three different human blank urines, and DPO in the spiked urine was extracted using immobilized SSA-Agarose gel. 173 to 282 mIU/mL of DPO was added. SSA-Agarose gel was suspended and allowed to stand for 1 to 66 hours. The recoveries 75.1 to 108.0% were obtained and better recovery of DPO was obtained by the shorter incubation time of 1 hour. Similar results were obtained also for FSH. Blank urine, blank urines spiked with the known amounts of recombinant FSH or FSH from human urine were extracted by immobilized SSA column. FSH in the eluates of the SSA column were quantitated by automated immunoassay AIA 600II and their glycan compositions were monitored by lectin microarray. Recovery of recombinant FSH was found to be 71.6 to 79.6% and no lectin interaction of extracted FSH in the recombinant fraction with SSA and SNA was observed.

Conclusions

Currently, doping with ESA is being confirmed by mobility or isoform compositions by immuno-blotting electrophoresis which recognizes the molecular size or the charge. For gonadal glycoprotein hormones, no test procedure is available for the origin identification, and test result is to be judged by referring the concentration to the applicable decision limits. Sometimes, the atypical test results needs to be followed by further longitudinal studies to eliminate a naturally elevated case caused by special physiological conditions, such as familial hCG [9]. All those decision making processes are time consuming. It is therefore considered that high-throughput origin specific assay would be one of the optimal goals of a doping test for glycoprotein hormones.

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