Monosaccharide composition analysis of glycoproteins by isotope-tag method and capillary LC/ESI-MS

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Abstract: Aim To develop a rapid and sensitive method for monosaccharide composition analysis. **Methods** Glycoprotein was first hydrolyzed to monosaccharides, which were subsequently reacetylated (amino monosaccharides), tagged with 2-aminopyridine and then separated and monitored in selected ion mode by CapGCC-LC/MS. The use of tetradeuterium labeled-aminopyridyl-monosaccharides prepared by tagging monosacahrides with hexadeuterium labeled 2-aminopyridine as internal standards improved the linearity and reproducibility in quantification. **Results** This method was successfully applied to monosaccharide composition analysis of model glycoproteins, fetuin and erythropoietin down to 1 pmol monosaccharides. **Conclusion** This method has been shown to be highly sensitive and is applicable to monosaccharide composition analysis of glycoproteins.

Key words: monosaccharide; pyridylamination; isotope-tag; LC/MS

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同位素标记结合毛细管液相色谱和电喷雾质谱分析糖蛋白单糖组成

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摘要: 目的 单糖组成分析能够为糖蛋白糖链结构的分析提供重要的信息。本文对一种高灵敏度及快速的单糖组成分析方法进行了研究。方法 糖蛋白通过酸水解释放出全部单糖,然后经过乙酰基化(氨基单糖),非氘取代2-氨基吡啶标识,毛细管石墨化碳柱分离,最终通过电喷雾质谱的可选择离子模式进行检测。由于分析中采用氘取代2-氨基吡啶标识的单糖作为内标,提高了单糖定量测定的准确性。结果 本方法可测定低至1皮摩尔的单糖,并已成功应用于牛胎球蛋白和促红细胞生成素单糖组成的分析。结论 本方法具有很高的灵敏度,可适用于微量糖蛋白单糖组成的分析。

关键词: 单糖; 吡啶标识; 同位素标记; 液相色谱 质谱

Glycoproteins are widely distributed in nature, and their exact carbohydrate contents are often an important feature in the study of their functional or biological properties. Monosaccharide composition analysis is very useful to determine the types of oligosaccharides (e. g. *O*-linked and *N*-linked; high mannose, and complex types) bound to a protein. Sensitive and selective methods are required for the monosaccharide composition analysis.

Many methods for the monosaccharide composition analysis in glycoproteins have been reported.

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Recently, high-performance anion-exchange chromatography with pulsed amperemetric detection (HPAEC-PAD) has been widely used for monosaccharide composition analysis[1-5]. It gives high resolution of all common monosaccharides and has the advantage of not requiring derivation and relatively high sensitivity (ca. 10 pmol), but it has limitation of selectivity. Colorimetric or fluorometric precolumn and postcolumn derivation techniques have been often used [6-11]. Precolumn derivation techniques can produce a chromaphore or fluorophore with high yield and have the distinct advantage of rendering sugar hydrophobicity, thus permitting the application of reversed phase HPLC. In these precolumn derivation methods, reductive pyridylamination has been widely used carbohydrate analysis for quantitative derivation.

Previously, we have demonstrated that capillary LC-MS equipped with graphitized carbon column (CapGCC-LC/MS) is useful for the analysis of oligosaccharides[12]. In this report, we describe a CapGCC-LC/MS based method for determination of monosaccharide composition of glycoproteins through reductive pyridylamination. We studied the use of deuterium-labeled PA (pyridylamino)-monosaccharides as internal standards. This method was successfully applied to monosaccharide composition analysis of model glycoproteins.

Materials and methods

Materials All PA-monosaccharide standards were obtained from TaKaRa Biomedicals (Otsu, Japan). All monosaccharide standards were bought from Seikagaku-kogyo (Tokyo, Japan). Pyridylamination apparatus (Palstation) was purchased from TaKaRa biomedicals. All chemicals and reagents necessary for pyridylamination reaction are also available as a kit from Takara Biomedicals. Hexadeuterium labeled (d_6)-2-aminopyridine was bought from Wako (Osaka, Japan). All other chemicals and reagents were of analytical grade and were commercially available.

Pyridylamination Free amino groups were acetylated by adding 50 μL of a mixture of methanol-pyridine-distilled water (30∶15∶10) and 2 μL of acetic anhydride. The solution was incubated for 30 min at room temperature with occasional stirring. The solution was dried using a vacuum centrifuge evaporator without heating, and to the residue was added 10 μL of coupling reagent prepared by mixing 100 mg of 2-aminopyridine, 50 μL of acetic acid, and 60 μL of methanol. The tube was heated at 90 ℃ for 20 min (Palstation, TaKaRa). The excess reagents were removed by evaporating under a stream of nitrogen gas at 60 ℃ for 20 min. Then 10 μL of a reducing reagent, prepared just before use by mixing 6 mg of borane-dimethylamine complex and 100 μL of acetic acid, was added and the tube was heated at 90 ℃ for 35 min. The reaction mixture was dried three times under a stream of nitrogen gas with 20 μL of methanol and 40 μL of toluene at 50 ℃ for 10 min. The residue was dissolved in water for LC/MS analysis. For the preparation of tetradeuterium labeled (d_4)-PA-monosaccharide isotope analogs, 2-aminopyridine was just replaced by d_6-2-aminopyridine.

LC/MS Analysis LC was carried out using a Magic 2002 HPLC system (Michrom Bioresources, Inc., Auburn, CA, USA). The column used was Hypercarb (0.2 mm × 150 mm, Thermoelectron, San Jose, CA, USA). The flow rate was set at 2 - 3 μL·min^{-1} through a splitter system. Mobil phases were 5 mmol·L^{-1} ammonium acetate (pH 8.5) with 2% of acetonitrile (pump A) and with 80% of acetonitrile (pump B). A gradient of 10 % to 35% of B in 60 min was used for the separation.

Mass spectrometer used was TSQ 7000 (Thermoelectron) equipped with a nanoelectrospray ion source (AMR, Inc., Tokyo, Japan). The mass spectrometer was operated on selective ion monitoring (SIM) mode in the positive-ion mode. The ESI voltage was set to 2 000 V, and the capillary temperature was 175 ℃.

Monosaccharide composition analysis of a glycoprotein A sample (25 pmol of a glycoprotein) was placed in a hydrolysis tube fitted with a Teflon-lined screw cap. To the sample was added 50 μL of 2 mol·L^{-1} HCl-2 mol·L^{-1} trifluoroacetic acid (TFA) and heated at 100 ℃ for 6 h. Simultaneously, a set of monosaccharide standards, 100 pmol of Gal (galactose), Man (mannose), Glc (glucose), Fuc (fucose), GlcN (glucosamine) and GalN (galactosamine), was treated identically as the samples. The solution obtained was freeze-dried. Monosaccharides obtained from the sample and standard were tagged with non-deuterium labeled (d_0)-2-aminopyridene and (d_4)-2-aminopyridene, respectively. Each tagged oligosaccharide mixture was dissolved into purified water, and 5 μL of each solution was combined. A mixture of d_0- and d_4-PA

monosaccharides (2 μL) was injected into CapGCC-LC/MS.

Results and discussion

1 Assessment of d_4-PA-monosaccharides as internal standard

Internal standard method is widely used to obtain a better precision in quantification. An isotope analog of analyte has been mostly used as internal standard in MS analysis. Because the deuterium isotope analogs of monosaccharides are not commercially available, we chose d_6-2-aminopyridine as labeling reagent. Four deuterium atoms were added to each PA (pyridylamino)-monosaccharide during pyridylamination reaction (Figure 1).

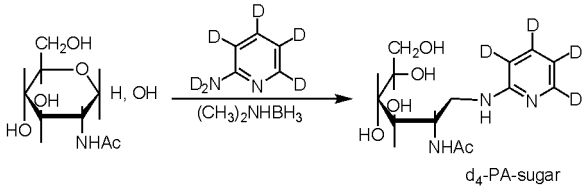

Figure 1 Synthesis of d_4-PA-monosaccharide internal standard

Due to the impurity of the reagents and the nature abundance of deuterium in the sample, a series of isotope analogs of PA-monosaccharides were produced, including d_2, d_3, d_4, d_5 and d_6-PA-monosaccharide (Table 1). Cross-contribution between the analyte and its labeled analog is a major factor affecting the accuracy achievable in a routine quantitative determination protocol[13], However, no evidence cross contribution between d_0-PA-monosaccharides and d_4-PA-monosaccharides was observed (Table 1).

2 Separation and detection of PA-monosaccharides

PA-monosaccharides (Gal, Man, Glc, Fuc, GlcNAc and GalNAc, 1 pmol each) of equal molar were analyzed by CapGCC-LC/MS in SIM (selected ion mode). The ions monitored were *m/z* 259 (for PA-Gal, PA-Man and PA-Glc), *m/z* 243 (PA-Fuc) and *m/z* 300 (PA-GlcNAc and PA-GalNAc). Figure 2A shows the TIC (total ion current) chromatogram of the d_0-PA-monosaccharides. All six PA-tagged monosaccharides were nearly baseline resolved with less than 2 min of separation in retention time. The peaks for all monosaccharides were sharp and nearly symmetrical. The detection limit at a signal-to-noise ratio of 3 was 0.045 pmol.

D_4-PA-monosaccharides were prepared with d_6-2-aminopyridine and combined with commercially available d_0-PA-monosaccharides, and the mixture was injected into CapGCC-LC/MS. The ions at *m/z* 263 (d_4-PA-Gal, d_4-PA-Man and d_4-PA-Glc), *m/z* 247 (d_4-PA-Fuc) and *m/z* 304 (d_4-PA-GlcNAc and d_4-PA-GalNAc) were monitored in addition to *m/z* 243, 259, and 300. Figure 2B and Figure 2C show the mass chromatograms of d_4-PA-monosaccharides and d_0/d_4-PA-monosaccharides, respectively. Paired ions with a difference of 4Da were detected in individual peaks in Figure 2C (Figure 2D-2I).

Different amounts of PA-monosaccharides were quantified using 1 pmol d_4-PA-sugars as internal standard. A linear relationship between the amount of PA-monosaccharide and the peak area ratio (d_0-PA-monosaccharides peak area/ d_4-PA-monosaccharides peak area) was observed over a range between 0.05-25 pmol of PA-monosaccharide. The reproducibility of the internal standard method was assessed by measuring RSD values for repeating assays of PA-monosaccharides (Table 2). The RSD values obtained with the isotope analog internal method were significantly lower than those obtained with rhamnose internal standard or those calculated without correction by internal standard.

Table 1 Evaluation of cross-contribution with direct measurement method

Ion (*m/z*)	SIM ion intensity/%		Ion (*m/z*)	SIM ion intensity/%						Ion (*m/z*)	SIM ion intensity/%			
	PA-Fuc	d_4-PA-Fuc		PA-Gal	d_4-PA-Gal	PA-Man	d_4-PA-Man	PA-Glc	d_4-PA-Glc		PA-GlcNAc	d_4-PA-GlcNAc	PA-GalNAc	d_4-PA-GalNAc
243	86.6		259	87.4		88.6		87.6		300	86.0		85.5	
244	11.7		260	11.0		9.9		11.1	1	301	12.3		12.9	
245	1.7	0.7	261	1.6	0.7	1.5	0.4	1.3	0.8	302	1.7	0.4	1.6	0.5
246		4.6	262		4.1		2.9		4.1	303		4.1		4.6
247		82.6	263		83.1		78.7		79.3	304		81.0		81.1
248		10.4	264		10.5		14.4		13.4	305		12.5		12.0
249		1.7	265		1.6		3.6		2.4	306		2.0		1.8

10 pmol d_0-PA-monosaccharides or d_4-PA-monosaccharide was injected. GalNAc: *N*-acetylgalactosamine; GlcNAc: *N*-acetyl glucosamine

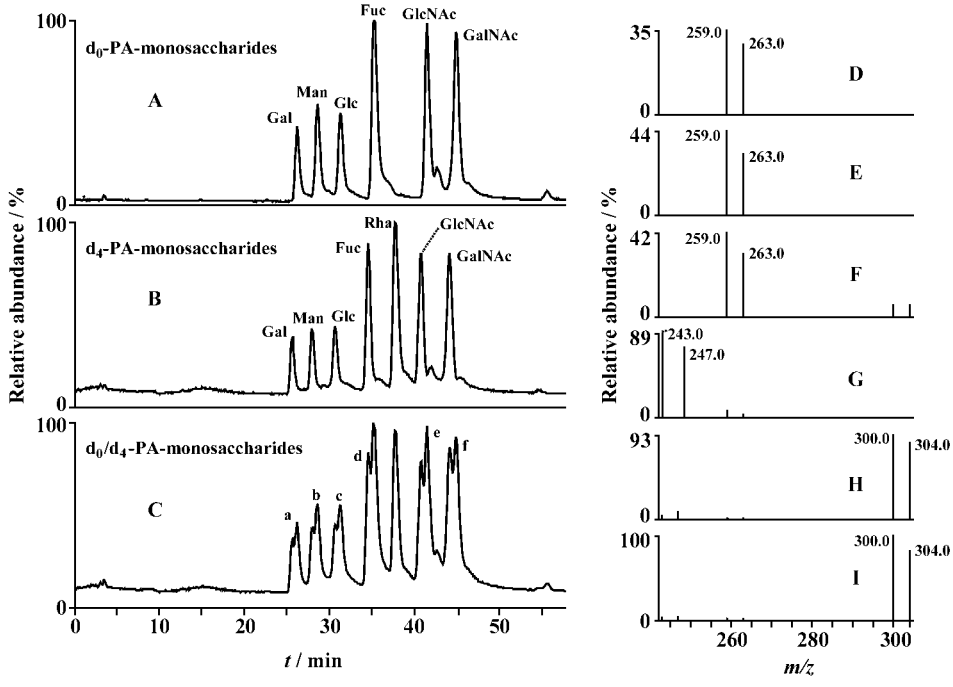

Figure 2　Mass chromatogram (set *m/z* 259, 243 and 300) of d_0-PA-monosaccharides (1 pmol Gal, Man, Glc, Fuc, GlcNAc and GalNAc) (A). Mass chromatogram (set *m/z* 263, 247 and 304) of d_4-PA-monosaccharides (1 pmol Gal, Man, Glc, Fuc, Rham, GlcNAc and GalNAc) (B). Mass chromatogram (set *m/z* 259, 263, 243, 247, 300 and 304) of a mixture of d_0- and d_4-PA-monosaccharides (1 pmol Gal, Man, Glc, Fuc, Rham, GlcNAc and GalNAc) (C) and mass spectra of peaks a (D), b (E), c (F), d (G), e (H) and f (I)

Table 2　RSD values for LC/MS of PA-monosaccharides with or without internal standard (n = 5)

PA-monosaccharide	PA-monosaccharides/pmol								
	0.05			0.5			5		
	IS(-)	IS(A)	IS(B)	IS(-)	IS(A)	IS(B)	IS(-)	IS(A)	IS(B)
Gal	16.2	15.5	5.7	11.6	8.3	2.1	5.0	5.5	2.3
Man	12.7	15.3	4.7	9.7	7.1	1.8	7.2	4.6	1.7
Glc	12.9	17.5	3.9	8.9	7.6	4.8	10.9	5.1	1.1
Fuc	23.9	17.3	4.2	9.9	7.6	1.9	5.4	6.1	0.8
GlcNAc	15.2	7.7	3.5	9.0	5.6	2.0	5.4	6.0	1.2
GalNAc	16.0	21.0	9.4	11.4	7.4	4.7	9.8	3.5	1.5

IS: Internal standard; (-): Without IS; A: PA-rhamnose; B: d_4-PA-monosaccharides

3　Quantification of monosaccharide using isotope-tag method

To assess the linearity of the isotope-tag method for quantification of monosaccharide, including reacetylation, pyridylamination, and removal of excess derivation reagents, different amounts of monosaccharides (Gal, Man, Glc, Fuc, GlcN and GalN) were tagged with d_0-2-aminopyridine. For the standards, 1 nmol monosaccharides were tagged with d_6-2-aminopyridine, and 4 pmol or 20 pmol d_4-PA-monosaccharides were co-injected with the d_0-PA-monosaccharides into CapGCC-LC/MS. The whole pyridylamination process was found to be linear for all six monosaccharides over the tested range of 1 pmol to 1 nmol. Accuracy of this method was approximately 80%-100%. The practical minimum amount of a glycoprotein required for pyridylamination and LC/MS analysis was found to be approximately 1 pmol. By using monosaccharide standard mixture, good reproducibility was obtained in this method. Relative standard deviations (RSD) of the whole process, including reacetylation, pyridylamination, removal of excess reagents and CapGCC-LC/MS analysis were less than 7.2% for all monosaccharides (based on the peak area ratio of monosaccharides from 5 samples).

4 Monosaccharide composition analysis of glycoproteins

Applications of this method for monosaccharide composition analysis of a glycoprotein were demonstrated using fetuin and erythropoietin as examples. Accuracy in monosaccharide composition analysis of a glycoprotein relied to the condition of hydrolysis. Fan *et al*[14] have studied hydrolysis of *N*-linked oligosaccharides and recommended 4 h with 2 mol• L^{-1} TFA at 100 ℃ for neutral sugars and 6 h with 4 mol• L^{-1} HCl at 100 ℃ for amino sugars. While these hydrolysis conditions give complete release of neutral and amino sugars with no degradation, it takes two hydrolysis for a single sample. To quantify the amount of neutral and amino sugars in glycoproteins at the same run, fetuin and erythropoietin (25 pmol) were heated in 2 mol• L^{-1} HCl-2 mol• L^{-1} TFA at 100 ℃ for 6 h was used[15]. Simultaneously, a set of monosaccharide standards, 100 pmol of Gal, Man, Glc, Fuc, GlcN and GalN, was treated identically as the samples. After hydrolysis, the samples and standards were tagged with d_0-2-aminopyridene and d_6-2-aminopyridene, respectively. Figure 3 shows the TIC chromatograms of monosaccharides from fetuin and erythropoietin. Curves 1, 3, and 5 show the mass chromatograms of samples (d_0-PA monosaccharides), and curves 2, 4, and 6 indicate the mass chromatograms of standards (d_4-PA-monosaccharides). Monosaccharide compositions of fetuin and erythropoietin calculated from peak area ratios (d_0-PA/d_4-PA-monosaccharides) were in good agreement with reported values[16-19] (Table 3). Decomposition of monosacchrides during hydrolysis could be corrected by heating standards simultaneously, and isotope analogs used as internal standards can improve deviation caused by MS analysis, thus monosaccharide composition was analyzed by using only small amount of samples.

Table 3 Monosaccahride composition of fetuin and erythropoietin determined by isotope-tag method

Glycoprotein	Monosaccharide	Ratio of mol[a]	Ratio of mol[b]
Fetuin	Fuc	0.3	0
	Gal	10.4	12
	Man	7.6	9
	GlcNAc	14.7	15
	GalNAc	3.4	3
Erythropoietin	Fuc	3.4	3
	Gal	12.8	13
	Man	8.1	9
	GlcNAc	15.6	18
	GalNAc	1.5	1

[a] Values were expressed as mol detected in 1 mol glycoprotein; [b] Expected values based on known composition

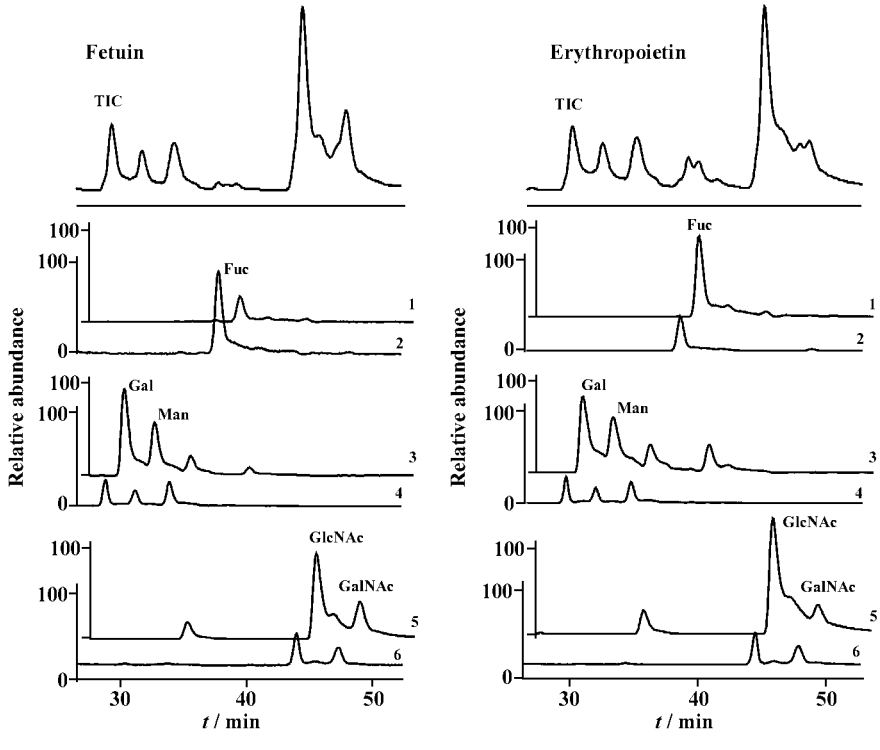

Figure 3 TIC chromatograms of monosaccharides from fetuin and erythropoietin. Mass chromatograms of d_0-PA-Fuc (m/z 243) (1), d_4-PA-Fuc (m/z 247) (2), d_0-PA-hexose (m/z 259) (3), d_4-PA-hexose (m/z 263) (4), d_0-PA-*N*-acetylhexosamine (m/z 300) (5), d_4-PA-*N*-acetylhexosamine (m/z 304) (6)

However, the amount of some monosaccharides detected in glycoproteins was less than expected values. It could be due to the complex structure of sugar chains. Because of their different place in sugar chains, the release efficiency of monosaccharides was different on the same hydrolysis condition. Those monosaccharides existing in the core of sugar chains were often more difficult to be released by acid hydrolysis. Although stronger acid such as 4 mol·L^{-1} HCl could be helpful, neutral sugars would be damaged largely on this condition. Therefore, a suitable hydrolysis condition is the key to obtain accurate monosaccharide composition. In addition, impure reagents will interfere analysis of monosaccharides. For example, microamount fucose was found in fetuin, it could be due to the contaminants in acid.

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References:

[1] Hardy MR. Monosaccharide analysis of glycoconjugates by high-performance anion-exchange chromatography with pulsed amperometric detection [J]. *Methods Enzymol*, 1989, **179**(1): 76 - 82.

[2] Ip CC, Manam V, Hepler R, *et al*. Carbohydrate composition analysis of bacterial polysaccharides: optimized acid hydrolysis conditions for HPAEC-PAD analysis [J]. *Anal Biochem*. 1992, **201**(2): 343 - 349.

[3] Lampio A, Finne J. Sugar analysis of glycoproteins and glycolipids after methanolysis by high-performance liquid chromatography with pulsed amperometric detection [J]. *Anal Biochem*, 1991, **197**(1): 132 - 136.

[4] Lee YC. High-performance anion-exchange chromatography for carbohydrate analysis [J]. *Anal Biochem*, 1990, **189**(2): 151 - 162.

[5] Weitzhandler M, Pohl C, Rohrer J, *et al*. Eliminating amino acid and peptide interference in high-performance anion-exchange pulsed amperometric detection glycoprotein monosaccharide analysis [J]. *Anal Biochem*, 1996, **241**(1): 128 - 134.

[6] Hase S, Hara S, Matsushima Y. Tagging of sugars with a fluorescent compound, 2-aminopyridine [J]. *J Biochem*, 1979, **85**(1): 217 - 220.

[7] Anumula KR, Taylor PB. Quantitative determination of phenyl isothiocyanate-derivatized amino sugars and amino sugar alcohols by high-performance liquid chromatography [J]. *Anal Biochem*, 1991, **197**(1): 113 - 120.

[8] Anumula KR. Quantitative determination of monosaccharides in glycoproteins by high-performance liquid chromatography with highly sensitive fluorescence detection [J]. *Anal Biochem*, 1994, **220**(2): 275 - 283.

[9] Lin JK, Wu SS. Synthesis of dabsylhydrazine and its use in the chromatographic determination of monosaccharides by thin-layer and high-performance liquid chromatography [J]. *Anal Chem*, 1987, **59**(9): 1320 - 1326.

[10] Honda S, Akao E, Suzuki S, *et al*. High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and electrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives [J]. *Anal Biochem*, 1989, **180**(20): 351 - 357.

[11] Fu D, O′Neill RA. Monosaccharide composition analysis of oligosaccharides and glycoproteins by high-performance liquid chromatography [J]. *Anal Biochem*, 1995, **227**(2): 377 - 384.

[12] Kawasaki N, Itoh S, Ohta M, *et al*. Microanalysis of *N*-linked oligosaccharides in a glycoprotein by capillary liquid chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry [J]. *Anal Biochem*, 2003, **16**(1): 15 - 22.

[13] Chang WT, Lin DL, Liu RH. Isotopic analogs as internal standards for quantitative analyses by GC/MS-evaluation of cross-contribution to ions designated for the analyte and the isotopic internal standard [J]. *Forensic Sci Int*, 2001, **121**(3): 174 - 182.

[14] Fan JQ, Namiki Y, Matsuoka K, *et al*. Comparison of acid hydrolytic conditions for Asn-linked oligosaccharides [J]. *Anal Biochem*, 1994, **219**(2): 375 - 378.

[15] Takemoto H, Hase S, Ikenaka T. Microquantitative analysis of neutral and amino sugars as fluorescent pyridylamino derivatives by high-performance liquid chromatography [J]. *Anal Biochem*, 1985, **145**(2): 245 - 250.

[16] Hardy MR, Townsend RR, Lee YC. Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection [J]. *Anal Biochem*, 1988, **170**(1): 54 - 62.

[17] Sasaki H, Ochi N, Dell A, *et al*. Site-specific glycosylation of human recombinant erythropoietin: analysis of glycopeptides or peptides at each glycosylation site by fast atom bombardment mass spectrometry [J]. *Biochemistry*, 1988, **27**(23): 8618 - 8626.

[18] Takeuchi M, Takasaki S, Miyazaki H, *et al*. Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells [J]. *J Biol Chem*, 1988, **263**(8): 3657 - 3663.

[19] Tsuda E, Goto M, Murakami A, *et al*. Comparative structural study of *N*-linked oligosaccharides of urinary and recombinant erythropoietins [J]. *Biochemistry*, 1988, **27**(15): 5646 - 5654.