http://dx.doi.org/10.5012/bkcs.2013.34.5.1571

# Method Optimization for Rapid Measurement of Carbohydrates in Plasma by Liquid Chromatography Tandem Mass Spectrometry

**Duc-Toan Nguyen,[a] Jundong Yu,[†,a] Sun-Il Mho,[‡] Hae-Lee Lee,[§] Wonjae Lee,[#] Sung-Tae Yee,[¶] Gwang Lee,[$] and Man-Jeong Paik[¶,*]**

*Department of Molecular Science and Technology, Ajou University, Suwon 443-749, Korea*
*[†]Racing Laboratory, Korea Racing Authority, Gwacheon 427-711, Korea*
*[‡]Department of Chemistry, Ajou University, Suwon 443-749, Korea*
*[§]Sangdosijang Pharmacy, Seoul 156-840, Korea*
*[#]College of Pharmacy, Chosun University, Gwangju 501-759, Korea*
*[¶]College of Pharmacy, Sunchon National University, Suncheon 540-950, Korea. [*]E-mail: paik815@sunchon.ac.kr*
*[$]Institute for Medical Science School of Medicine, Ajou University, Suwon 443-721, Korea*
*Received November 27, 2012, Accepted February 26, 2013*

**Key Words :** Glucose, Carbohydrate, Liquid chromatography-electrospray ionization mass spectrometry, Human plasma

Carbohydrates are one of the most abundant classes of organic compounds in nature, which not only constitute complex biomolecules in human and animals but are also distributed in plants and bacteria.[1,2] Among a variety of carbohydrates, glucose is a key compound in carbohydrate metabolism. In humans, glucose circulates in the blood and plays important roles through a variety of functions: glucose is not only the main source of energy for most organs such as muscles, central nervous system and brain but it also provides substrates to other metabolic reactions.[3,4] Separation and discrimination of carbohydrates and glucose levels in human matrices so thus have been continued growing more and more in carbohydrate metabolism research.[2,5-20] Recently, high performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS) has become a powerful and essential tool for trace determination of not only carbohydrates but also a variety of compounds due to its rapid, specificity, and sensitivity.[7,12-20] ESI involves transferring compounds from liquid solution to gas phase and the formation of charged ions under a strong electrical field. This soft ionization method accomplishes a number of processes including the production of charged droplets, desolvation, ion generation, declustering.[21,22] Hence, the sensitivity of ESI-MS is not only strongly dependent on the properties of analytes, samples preparation methods,[23] and chromatographic conditions,[23-27] but is also decided by series of its own parameters.[21-27] These factors can enhance or suppress the ion intensity, resulting in the enhancement or suppression of signal for analytes; therefore these effects should be examined in order to achieve optimal conditions and improve the sensitivity of the method.[23-27] This report presents the systematic optimization of both ESI source and analyte-dependent parameters for analysis of a variety of carbohydrates by LC-MS.

[a]These authors contributed equally to this work.

## Experimental Section

Seven carbohydrates (xylose, fucose, fructose, glucose, sucrose, cellobiose, melezitose), internal standard (salicin) and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile (ACN) were pesticide grade and obtained from Kanto Chemical (Chuo-ku, Tokyo, Japan). Formic acid and acetic acid were purchased from Fluka (St. Louis, MO, USA). Deionized water (resistivity more than 18 MΩ) was produced by an Ultra-pure water system from Sinhan science Tech Co. Ltd. (Yuseong-gu, Dejeon, Korea).

LC analysis was carried out using a Dionex Ultimate 3000 liquid chromatography system (Dornierstraße 4, D-82110 Germering, Germany) consisting of a Dionex SRD-3600 vacuum degasser, a LPG-3600 micro pump, a FLM-3100 flow manager and a WPS-3000T auto-sampler. Carbohydrates were separated using a Luna amino column (150 mm × 2 mm ID., 3 μm particle size) from Phenomenex (Torrance, CA, USA) at a flow rate of 200 μL/min. The column temperature was maintained at 40 °C. Sample volumes of 5 μL were introduced by partial mode. The mobile phase was composed of acetonitrile (A) and water (B) with gradient program as follows: 0-6 min, 80% A + 20% B; 6-8 min, 80% A + 20% B to 60% A + 40% B and maintained for 4 min (8-12 min); finally, the column was equilibrated for 13 minutes (12.1-25 min) with 80% A + 20% B.

ESI-MS experiments were performed on a MDS SCIEX 3200 QTRAP tandem mass spectrometer (Applied Biosystems, Toronto, Canada) equipped with a turbo ionspray source. The ion spray voltage was set to –4500 V, the probe temperature was 500 °C with the interface heater turned on. High-purity nitrogen gas and air supplied from a Peak scientific gas generator (Inchinnan, PA4 9RE, Scotland) were used as auxiliary gas for curtain, nebulization and vaporization processes. The ESI-MS was operated in negative ion

mode. The compound-dependent parameters including declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP) were individually optimized for all compounds by flow injection analysis (Table 1). For these experiments, an HPLC pump was used at a flow rate of 0.2 mL/min with an eluent of acetonitrile-water (80:20, v/v), and 5 μL of standards at 0.1 mg/mL were injected directly into the MS without a column. Both quadrupole Q1 and Q3 were set to unit resolution. The deprotonated molecule $[M-H]^-$ ions were monitored in selected ion monitoring mode (SIM) and were chosen as the precursor ions for two specific multiple reaction monitoring (MRM) transitions in MS/MS mode. Data acquisition and quantification were performed using Analyst 1.4 software (Applied Biosystems, Toronto, Canada).

Standard stock solutions of seven carbohydrates (xylose, fucose, fructose, glucose, sucrose, cellobiose, melezitose) were individually prepared at 10 mg/mL in deionized water. Working standard solutions and standard mixtures (100, 10 and 1.0 μg/mL) were made up from stock solutions by further dilution with ACN-deionized water (80:20, v/v). A solution of salicin at 0.1 mg/mL in 80% ACN solution was prepared from its stock solution (10 mg/mL) and used as the internal standard (IS) since it is not found in human biological samples.

Plasma samples were collected from healthy volunteers and stored in a deep freezer at −79 °C until use. Plasma samples were thawed at room temperature and 10 μL were spiked with 25.0 μL of internal standard (100 μg/mL) and vortex-mixed for 30 seconds. Plasma proteins were precipitated by addition of ACN-water (80:20, v/v) mixture (965 μL), then vortex-mixed for 3 min and centrifuged at 14000 rpm for 10 min. The supernatant was filtered through 0.2 μm membrane, diluted 5-fold with a mixture of ACN-water (80:20, v/v) and transferred to auto-sampler vials. 5 μL of the diluted plasma extract was injected onto the LC tandem MS system.

Calibration standards ranged from 0.005 to 5.0 μg/mL (0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0), each containing 0.5 μg/mL of IS, were prepared from the standard mixture in 80% ACN. Linear regression of peak area ratios of analytes/internal standard against corresponding concentrations were used to plot the calibration curve. The slope, intercept and correlation coefficient values were established from the regression line and used for quantification. Intra-assay precision and accuracy were examined at three different levels.

## Results and Discussion

In this method, clean-up is a necessary and important process for sample preparation in order to prevent blockage in the HPLC column and ESI source, which is based on protein precipitation with acetonitrile (ACN) and subsequent centrifugation and membrane filtration for removal of insoluble particles before injection into the LC-MS system. The advantage is that sample preparation reduces time to a minimum and enables direct injection of the diluted plasma extract into the LC-MS system.[7] This procedure is simple and rapid, consumes minimal solvent and yields pure carbohydrates from plasma samples for biological analysis.

**Optimization of the LC Separation.** Mobile phases composed mainly of ACN and water provided good separation and reasonable retention time for glucose and several carbohydrates on an amino column.[11,14,15] In this work, a mobile phase composed of ACN and water without any modifier was used. The retention of carbohydrates on a bonded aminopropyl silica column could be controlled by the proportion of organic modifier in the mobile phase because the retention is based on the hydrophilic interaction between the carbohydrate hydroxyl groups and the stationary phase.[5,6] In the present study, different percentages of ACN in the mobile phase were tested. By using isocratic flow, increases in peak broadening and analysis time were observed when the organic content increased from 60 to 85%, while the resolution of carbohydrates was not improved. When simultaneously analyzing glucose together with di- and tri saccharides, the initial isocratic flow of 80% ACN was maintained for the first six minutes and then modified to a gradient program by decreasing the ratio of ACN from 80 to 60%. This resulted in a well-resolved chromatogram with appropriate retention times and improved the peak shape for all compounds.

The effect of flow rate on the signal response of $[M-H]^-$ ions for carbohydrates was investigated. The intensities of $[M-H]^-$ ions decreased with the increase of flow rate from 200-500 μL/min; lower flow rate gave higher sensitivity in the same ESI-MS conditions. Flow rate reduces the size of charged droplets that minimize the solvent cluster, and less solvent vaporization is therefore required prior to transfer into the gas phase.

The signal suppression/enhancement of ESI-MS may occur due to changes in these conditions. Several additives, such as formic acid, acetic acid, and ammonium acetate at concentrations ranging from 1 to 10 mM, were employed in negative mode for investigation of the effect of modifiers on the ESI-MS response. Mass spectrometric properties and the specific fragmentation of each compound in negative mode were confirmed, and the mobile phase additives enhanced the intensities of adduct ions $[M+HCOO]^-$, $[M+CH_3COO]^-$ for all compounds tested in negative mode. The ratio of $[M+HCOO]^-/[M-H]^-$ was significantly increased when formic acid was added, while $[M+CH_3COO]^-/[M-H]^-$ was increased in the presence of acetic acid or ammonium acetate. However, the intensities of these adduct ions were relatively weak compared to those of the corresponding deprotonated molecular ions $[M-H]^-$; hence, the prominent $[M-H]^-$ ions of carbohydrates were monitored in Q1 SIM and used as precursor ions for multiple reaction monitoring (MRM) transitions.

Sodium adduct molecular ions $[M+Na]^+$ were observed in positive mode as the most abundant ions for all examined analytes since carbohydrates are not easily protonated to form $[M+H]^+$ ions. The formation of a sodium adduct in

positive ionization without the addition of sodium salt to the mobile phase also gave higher intensity than $[M-H]^-$ ions in negative mode at the same concentration level. However, the trace amounts of sodium originating from various sources such as mobile phase impurities, glassware, and analytical instruments may vary and thus affect the response of ions. Therefore, deprotonated molecular ions were selected for analysis of carbohydrates in negative ion mode. After optimization of the chromatographic separation, the MS parameters were optimized by flow injection analysis to achieve the maximum response for each analyte. The source-dependent parameters include ion spray voltage, temperature (TEM), gas 1 (GS 1) and gas 2 (GS 2). Ion spray voltage depends on the polarity and affects the stability of the spray and the sensitivity.[28] The response of $[M-H]^-$ ions of carbohydrates was affected by the electro spray voltage. Electro spray voltage was examined from −2000 to −4500 V for all analytes. A higher ion spray voltage drastically increased the signal intensity, and the optimal value was set at −4500 V. Gas 1 and gas 2 control the nebulizer gas and auxiliary gas, respectively. The signal intensity was enhanced by increasing GS 1 and GS 2 values from 0 to 60 psi, depending on the compounds. The response tended to be reduced at too higher pressure of GS 1 and GS 2 (data not shown). The results demonstrate that ion spray voltage, TEM, GS 1 and GS 2 are source-dependent parameters that can affect the response of ions in the ESI-MS; that is, they depend on the LC conditions and should be optimized to significantly impact the sensitivity of the analysis.

Declustering potential (DP) is used to minimize solvent cluster ions that may attach to the sample. The higher the voltage, the greater the amount of fragmentation or declustering, however, too high DP value can cause ion suppression and unexpected fragmentation of analytes. The intensities of $[M-H]^-$ ions of the studied carbohydrates increased gradually with the increase of DP values at low range to reach the maximum response; after passing this optimal point, the signal decreased with the higher DP values. Entrance potential (EP) guides and focuses the ions through the high pressure region and affects the values of ion path voltage. An acceptable value of EP equal to −10 V is recommended in negative mode for nearly all applications;[28] however in this study, lower EP values indicated that carbohydrates may involve fragile analytes (Table 1). The MRM transition ion for each carbohydrate is shown in Table 1. In MS/MS-type experiments, collision energy (CE), collision cell entrance potential (CEP), and collision cell exit potential (CXP) control the energy, entrance potential and exit potential of collision cell, respectively.[28] CE provides energy to the precursor ions transmitted the collision cell where they collide with gas molecules and fragment, while CEP and CXP are used to focus and accelerate ions. The effect of CE on the ESI response was investigated from −5 to −70 V. The parent ions may not fragment at initial low CE. Among the examined carbohydrates, sucrose and melezitose required the highest energy to collide in the collision cell. The signal intensity increased significantly with higher CE, but drastically decreased with too high CE. The reduction of response and increase in background noise may be due to the excessive fragmentation of precursor ions when too much energy was provided.

**Table 1.** Optimal ESI-MS parameters for MRM experiments

| Analyte | Molecular mass | MRM transition | DP | EP | CE | CEP | CXP |
|---|---|---|---|---|---|---|---|
| Xylose | 150 | 149/89 | -20 | -1 | -10 | -5 | -5 |
| Fucose | 164 | 163/89 | -15 | -1 | -10 | -5 | -5 |
| Fructose | 180 | 179/89 | -20 | -3 | -10 | -10 | -5 |
| Glucose | 180 | 179/89 | -20 | -10 | -10 | -10 | -5 |
| Sucrose | 342 | 341/89 | -40 | -4 | -30 | -15 | -5 |
| Cellobiose | 342 | 341/161 | -15 | -3 | -10 | -10 | -5 |
| Melezitose | 504 | 503/323 | -40 | -10 | -30 | -20 | -5 |
| Salicin (IS) | 286 | 285/123 | -40 | -10 | -10 | -10 | -5 |

Results from calibration curves are summarized in Table 2. The correlation coefficients were better than 0.999 over the range of 0.005-5 μg/mL for all carbohydrates examined. The limit of detection (LOD) for each carbohydrate was evaluated based on the signal of the blank and the standard deviation of the sample that gave a significantly different signal from the blank. LOD was calculated as 3 times the standard deviation divided by the slope of triplicate measurements of spiked sample at 50 ng/mL and 10 ng/mL for SIM and MRM mode, respectively. The method presents high sensitivity for carbohydrates with LOD ranging from 4.1-11.7 ng/mL in SIM mode and 1.4-5.9 ng/mL in the MRM

**Table 2.** Calibration data of carbohydrates by selected ion monitoring method

| Analyte | Diagnostic ion (*m/z*) | LOD[a] (ng/mL) | Amount added (μg) | Precision (% RSD)[b] | Accuracy (% RE)[c] |
|---|---|---|---|---|---|
| Xylose | 149 | 8.6 | 0.1 | 3.4 | -0.2 |
| | | | 0.5 | 4.9 | 7.8 |
| | | | 2.0 | 2.9 | -0.6 |
| Fucose | 163 | 7.1 | 0.1 | 2.2 | 10.9 |
| | | | 0.5 | 2.5 | 3.4 |
| | | | 2.0 | 4.2 | -1.0 |
| Fructose | 179 | 8.6 | 0.1 | 0.5 | 3.1 |
| | | | 0.5 | 6.9 | 9.7 |
| | | | 2.0 | 5.7 | 2.6 |
| Glucose | 179 | 4.1 | 0.1 | 7.9 | 10.6 |
| | | | 0.5 | 2.9 | 0.1 |
| | | | 2.0 | 2.8 | -1.1 |
| Sucrose | 341 | 11.7 | 0.1 | 4.0 | 8.6 |
| | | | 0.5 | 4.9 | 8.8 |
| | | | 2.0 | 6.3 | 2.0 |
| Cellobiose | 341 | 4.2 | 0.1 | 8.9 | 7.3 |
| | | | 0.5 | 5.7 | 2.6 |
| | | | 2.0 | 5.9 | -4.6 |
| Melezitose | 503 | 10.1 | 0.1 | 4.2 | -3.9 |
| | | | 0.5 | 9.2 | 5.5 |
| | | | 2.0 | 3.7 | 3.1 |

[a]Limit of detection was calculated based on the response of spiked sample at 100 ng/mL. [b]Relative standard deviation. [c]Relative error

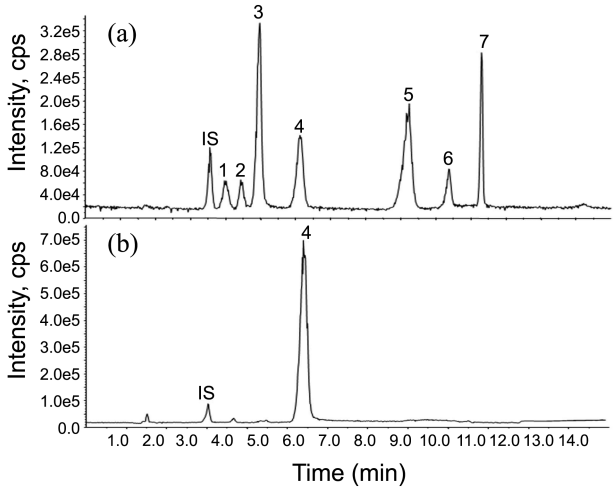


**Figure 1.** SIM chromatograms of carbohydrates (a) and plasma sample (b). **1**, fucose; **2**, xylose; **3**, fructose; **4**, glucose; **5**, sucrose; **6**, cellobiose; **7**, melezitose, **IS**, salcin.

experiment. Typical SIM chromatograms of carbohydrates and glucose in plasma samples are shown in Figure 1.

The precision expressed as percentage relative standard deviation (% RSD), was calculated from triplicate experiments. The accuracy was measured as the percentage difference (relative error, % RE) from theoretical added values obtained from three samples spiked with standards. The method showed acceptable precision (% RSD = 0.5-9.2), and accuracy (% RE = −4.6-10.9) for all analytes at three concentration levels (Table 2) and was reliable for the quantitative measurement of carbohydrates in biological samples. The validated method was applied for the measurement of glucose in human plasma. The mean of glucose concentration in normal subjects was $0.89 \pm 0.15$ mg/mL (0.68-1.15 mg/mL). These were similar with those reported other methods.[7-9] The presence of fructose in human plasma was also confirmed.[10] Other carbohydrates were not found in human plasma or their concentrations were below the LOD of the method.

## Conclusions

In conclusion, the developed HPLC coupled with ESI-MS was a powerful technique for the separation and characterization of carbohydrates by either SIM or MRM mode. The present method will be useful for the monitoring of carbohydrate profile in biological fluids from various diseases including diabetic ketoacidosis, hypoglycemia and hyperosmolar coma.

**Acknowledgments.** This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (2010-0021443, 2012R1A1A2007846, 2012M1A2A2671691) and by Suncheon Research Center for Natural Medicines.

## References

1. Sanz, M. L.; Martínez-Castro, I. *J. Chromatogr. A* **2007**, *1153*, 74.
2. Lopes, J. F.; Gaspar, E. M. S. M. *J. Chromatogr. A* **2008**, *1188*, 34.
3. Garrett, R. H.; Grisham, C. M. *Biochemistry*; Harcourt Brace, 2nd ed.; 1999.
4. Dwyer, D. S. *Glucose metabolism in the brain*; Academic Press: New York, 2002.
5. Churms, S. C. *J. Chromatogr. A* **1996**, *720*, 75.
6. Ikegami, T.; Tomomatsu, K.; Takubo, H.; Horie, K.; Tanaka, N. *J. Chromatogr. A* **2008**, *1184*, 474.
7. McIntosh, T. S.; Davis, H. M.; Matthews, D. E. *Anal. Biochem.* **2002**, *300*, 163.
8. Schierbeek, H.; Moerdijk-Poortvliet, T. C. W.; van den Akker, C. H. P.; te Braake, F. W. J.; Boschker, H. T. S.; van Goudoever, J. B. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 3824.
9. Magni, F.; Parom, R.; Bonini, P. A.; Kienle, M. G. *Clin. Chem.* **1992**, *38*, 381.
10. Wahjudi, P. N.; Patterson, M. E.; Lim, S.; Yee, J. K.; Mao, C. S.; Lee, W. N. P. *Clin. Biochem.* **2010**, *43*, 198.
11. Estevinho, B. N.; Ferraz, A.; Rocha, F.; Santos, L.; Alves, A. *J. Sep. Sci.* **2009**, *32*, 3116.
12. Bruggink, C.; Maurer, R.; Herrmann, H.; Cavalli, S.; Hoefler, F. *J. Chromatogr. A* **2005**, *1085*, 104.
13. Antonio, C.; Pinheiro, C.; Chaves, M. M.; Ricardo, C. P.; Ortuòo, M. F.; Thomas-Oates, J. *J. Chromatogr. A* **2008**, *1187*, 111.
14. Rogatsky, E.; Jayatillake, H.; Goswami, G.; Tomuta, V.; Stein, D. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1805.
15. Yuan, J.; Hashii, N.; Kawasaki, N.; Itoh, S.; Kawanishi, T.; Hayakawa, T. *J. Chromatogr. A* **2005**, *1067*, 145.
16. Rogatsky, E.; Stein, D. *J. Chromatogr. A* **2005**, *1073*, 11.
17. Liu, Y.; Urgaonkar, S.; Verkade, J. G.; Armstrong, D. W. *J. Chromatogr. A* **2005**, *1079*, 146.
18. Nielsen, N. J.; Granby, K.; Hedegaard, R. V.; Skibsted, L. H. *Anal. Chim. Acta* **2006**, *557*, 211.
19. Wan, E. C. H.; Yu, J. Z. *J. Chromatogr. A* **2006**, *1107*, 175.
20. Guignard, C.; Jouve, L.; Bogéat-Triboulot, M. B.; Dreyer, E.; Hausman, J. F.; Hoffmann, L. *J. Chromatogr. A* **2005**, *1085*, 137.
21. Kebarle, P. *J. Mass Spectrom.* **2000**, *35*, 804.
22. Manisali, I.; Chen, D. D. Y.; Schneider, B. B. *Trends Analyt. Chem.* **2006**, *25*, 243.
23. Mallet, C. R.; Lu, Z.; Mazzeo, J. R. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 49.
24. García, M.C. *J. Chromatogr. B* **2005**, *825*, 111.
25. Constantopoulos, T. L.; Jackson, G. S.; Enke, C. G. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 625.
26. Gao, S.; Zhang, Z. P.; Karnes, H. T. *J. Chromatogr. B* **2005**, *825*, 98.
27. Amad, M. H.; Cech, N. B.; Jackson, G. S.; Enke, C. G. *J. Mass Spectrom.* **2000**, *35*, 784.
28. *3200 Q TRAP LC/MS/MS Operator´s Manual*, Applied Biosystems/ MDS SCIEX, Ontario, 2005.