Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 839 (2006) 124–129
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Determination of iohexol clearance by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)☆

Soo-Youn Lee [a], Mi-Ryung Chun [a], Dae-Joong Kim [b], Jong Won Kim [a,*]

[a] *Department of Laboratory Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea*
[b] *Department of Internal Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea*

Received 12 September 2005; accepted 30 March 2006
Available online 3 May 2006

Abstract

We have developed a simple, rapid, and accurate HPLC-MS/MS method for the determination of iohexol in serum. The column used was a Zorbax Eclipse XDB-C8 (100 mm × 2.1 mm i.d., 3.5 μm). Mobile phases consisted of water containing 2 mM ammonium acetate and 0.1% formic acid (A) and methanol containing 2 mM ammonium acetate and 0.1% formic acid (B). After simple protein precipitation with $ZnSO_4$, serum samples were mixed with I.S. (bromperidol) and centrifuged for 3 min. The obtained extraction recovery at three levels was 94.6–107.4%. Quantitative analysis was performed in the multiple reaction-monitoring mode (*m/z* 822.0 → 804.0 for iohexol, 420.1 → 122.7 for I.S.) with the total running time of 3 min for each sample. The assay was linear between 0.5 and 1500 μg/mL ($r^2 > 0.997$). The intra- and inter-assay coefficient of variations were 2.4 – 6.2% and 5.5 – 6.5%, respectively. Our method provided sufficient analytical range and specificity for the 210 clinical samples analyzed.

Keywords: Iohexol clearance; HPLC-MS/MS

1. Introduction

Iohexol (*N*,*N*′-bis(2,3-dihydroxypropyl)-5-[*N*-(2,3-dihydroxypropyl)-acetamido]-2,4,6-triiodo-isophthalamide) is an iodinated, water-soluble, nonionic monomeric contrast medium with a molecular weight of 821.14 [1]. Iohexol is a suitable marker for glomerular filtration rate (GFR) because its elimination occurs by glomerular filtration without tubular secretion or resorption.

Use of iohexol for the determination of GFR has gained increasing acceptance as an alternative to the use of radioactive markers, such as ^{51}Cr-EDTA, ^{99m}Tc-DPTA or ^{125}I-iothalamate. Iohexol clearance is considered to be almost as accurate as ^{51}Cr-EDTA clearance [2] and agrees closely with inulin clearance [3,4], which remains the gold standard for GFR measurement. Moreover, GFR measurement using iohexol clearance from only one or two blood samples obtained at a fixed time have shown satisfactory results [3–5]. Therefore, GFR determination using iohexol clearance is widely used in clinical practice.

Several HPLC-UV methods to determine serum iohexol concentrations have been introduced [1,2,4,6–8]. A colorimetric method including deiodination of iohexol by alkaline hydrolysis and measurement of released iodide by the ceric arsenite method [9], X-ray fluorescence [2], capillary electrophoresis [10], and inductively coupled plasma-atomic emission spectroscopy [11] have also been employed for determination of iohexol.

For routine use in the clinical setting, the analytical method should be simple, rapid, and cost-effective. The HPLC-MS/MS method for the measurement of iohexol would provide advantage over other methods that require considerable time and labor for GFR measurement. Here we introduced the HPLC-MS/MS method for the determination of iohexol clearance and measured iohexol concentrations in five healthy subjects and ten diabetic patients with impaired renal function.

☆ This paper was presented at the 4th International Symposium on Separations in the BioSciences (SBS '05), Utrecht, The Netherlands, 18–21 September 2005.

* Corresponding author at: Department of Laboratory Medicine, Samsung Medical Center, #50, Ilwon-dong, Kangnam-ku, Seoul 135-710, South Korea. Tel.: +82 2 3410 2705; fax: +82 2 3410 2719.
E-mail address: jwonk@smc.samsung.co.kr (J.W. Kim).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.03.057

2. Experimental

2.1. Analytical method and validation

Iohexol (99.9% pure) was purchased from Promochem (Rockville, MD, USA). I.S. (bromperidol, 99.8% pure) was kindly donated by Hanhwa Pharmaceuticals (Seoul, South Korea). Ultra-pure water was obtained using a Milli-Q purification system (Milipore, Molsheim, France) and HPLC-grade acetonitrile, methanol, formic acid, and ammonium acetate were obtained from Sigma (Sigma, Missouri, USA).

Calibration standards containing 0, 10, 100, 250, 500, 750 and 1500 μg/mL of iohexol were prepared in duplicate daily for each analytical batch. Ten microliters of serum sample or calibrator was added to 40 μL of 0.1 M $ZnSO_4$ and vortexed for 30 s. We added 100 μL of I.S. solution (10 ng/mL bromperidol in acetonitrile) to the mixture. After vortexing for 30 s, the mixture was centrifuged for 3 min at 20817 × *g* (14,000 rpm). Ten microliters of the supernatant was directly injected onto the analytical column.

Analyses were performed on a Quatromicro API tandem mass spectrometer (Waters, Manchester, UK) equipped with Waters 2795 Alliance HPLC. Gradient chromatographic separation was performed on a Zorbax Eclipse XDB-C8 (100 mm × 2.1 mm i.d., 3.5 μm, Agilent, Wilminton, DE, USA), which was maintained at a temperature of 45 °C. The mobile phase comprised of water containing 2 mM ammonium acetate and 0.1% formic acid (mobile A) and methanol containing 2 mM ammonium acetate and 0.1% formic acid (mobile B). The gradient program, flow rate and mobile phase composition are shown in Table 1. The mass spectrometer was operated in positive ion detection mode. Nitrogen was used as the desolvation gas (650 mL/min). The source and desolvation temperatures were set at 120 and 320 °C, respectively. For collision-induced dissociation (CID), Argon was used as the collision gas at a pressure of 3.5×10^{-3} mbar. The mass detector tune setting are shown in Table 1. Analytes were monitored in the multiple reaction monitoring (MRM) mode using mass transition $[M+H]^+$ to product ions 822.0 → 804.0 for iohexol and 420.1 → 122.7 for I.S. Quantification was performed using integrated peak area ratio of iohexol to I.S. and calculated with Masslynx 4.0 software (Waters, Manchester, UK).

Table 1
HPLC-MS/MS operating conditions

HPLC gradient condition

Time (min)	Mobile A (%)	Mobile B (%)	Flow rate (mL/min)	
0.0	70	30	0.4	Start
0.2	0	100	0.4	
1.4	70	30	0.4	
3.0	70	30	0.4	End

MS/MS condition
MRM acquisition

MRM *m/z* pairs		Dwell (s)	Cone (V)	Collision (eV)
Iohexol	822.0 > 804.0	0.2	28	25
IS	420.1 > 122.7	0.2	30	45

MS/MS condition
Tune setting

LM1	HM1	LM2	HM1	Source (°C)	Desolvation (°C)	Capillary (kV)
14	14	13.5	13.5	130	300	3.2

Precision and accuracy were assessed by adding iohexol into pooled serum at three concentration levels (10, 100 and 500 μg/mL) using five replicates at each level for iohexol for five independent assay runs.

To evaluate linearity, calibration curves were prepared and assayed in duplicate on 5 consecutive days for iohexol concentrations up to 1500 μg/mL. The lower limit of quantification was defined as the lowest concentration with a signal-to-noise ratio of >10:1 and with a precision of coefficient of variation (CV) <15% from the analysis of 10 replicates.

The extraction recovery of iohexol was determined at three different concentrations (10, 100 and 500 μg/mL). The recovery of I.S. was also evaluated by serum samples of two concentrations (10 and 100 μg/mL). The concentrations of spiked serum samples were compared with those of standards prepared in mobile phase. The stability of iohexol was evaluated for 24 h at room temperature, −20 °C for 2 months, at the end of three freeze–thaw cycles and in stock solution. To test the post-preparative stability, the prepared samples were placed into the autosampler at 10 °C for 6 h.

The ion suppression effect was investigated using pooled blank serum sample, injected in the LC–MS/MS system after protein precipitation, in parallel with continuous post-column infusion of iohexol and I.S. in the electrospray source.

2.2. Determination of serum iohexol in the clinical specimens

This study was approved by the ethics committee. Five healthy donors for kidney transplantation and 10 diabetic patients with chronic renal failure were included. Serum creatinine and measured creatinine clearance (from a 24 h urine collection and serum creatinine) were 0.8–1.0 mg/dL and 114.9 ± 45.7 mL/min, respectively, in healthy subjects and 1.3–8.0 mg/dL and 20.6 ± 17.6 mL/min (range: 4.9–56.7 mL/min) in patients. The patients had been taking a variety of medications such as oral hypoglycemic agents, lipid-lowering agents, anti-hypertensives, and diuretics.

Participants were given a single injection (0.1 mL/kg) of iohexol solution (Omnipaque™ 300, 300 mg/mL, Amersham Health, Cork, Ireland) over 2 min followed by 10 mL of saline solution. In total, 210 blood samples were collected for the determination for serum iohexol concentration for up to 5 h after iohexol administration (0, 5, 10, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240 and 300 min) in five healthy subjects and up to 12 h (0, 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 300, 480 and 720 min) in patients.

Table 2
Precision and accuracy obtained for iohexol in serum

Added concentrations (μg/mL)	Number of observations	Measured concentrations			
		Mean (μg/mL)	S.D. (μg/mL)	CV (%)	Bias (%)
Inter-assay					
10	5	10.1	0.6	6.0	1.0
100	5	102.7	6.7	6.5	2.7
500	5	492.4	27.1	5.5	−1.5
Intra-assay					
10	5	10.3	0.6	5.8	3.0
100	5	91.5	4.2	6.2	−8.5
500	5	481.3	11.6	2.4	−3.7

Table 3
Extraction recoveries of iohexol and I.S. from serum

Added concentration (μg/mL)	Number of observations	Measured concentration (μg/mL)	Recovery (%)
Iohexol			
10	5	9.5 ± 0.6	94.6
100	5	107.4 ± 1.0	107.4
500	5	480.9 ± 6.1	96.2
I.S.			
10	5	9.6 ± 1.9	96.0
100	5	99.8 ± 2.3	99.8

3. Results

Representative ion chromatograms are shown in Fig. 1. Total run time was 3 min with retention time of 0.7 min for iohexol and 1.5 min for I.S.

The calibration curve provided a linear response up to the iohexol concentration of 1500 μg/mL ($n = 5$, slope = 0.0056 ± 0.0015, intercept = 0.1031 ± 0.4108, $r^2 = 0.9979 \pm 0.0013$). The lower limit of quantification for iohexol was 0.5 μg/mL (CV 13.5%, $n = 10$). The accuracy was satisfactory for all tested concentrations (Bias −8.5 to +3.0%). The intra- and inter-assay CVs were 2.4–6.2% and 5.5–6.5%, respectively, for the three levels of iohexol (Table 2).

Extraction recovery of iohexol was estimated to be 94.6–107.4% and that of I.S. was more than 95% as shown in Table 3. In all stability tests, the analytes were considered stable, as the measured concentration after the treatment was −5.4 to +5.6% of the nominal value (Table 4).

The analysis of pooled iohexol-free serum and blank samples from patients before iohexol injection showed no interfering peaks at the retention times of iohexol and I.S. No ion suppression effects were observed at the retention times of the two analytes (Fig. 2).

The maximal iohexol concentrations were 819.6 μg/mL in healthy subjects and 1358.3 μg/mL in diabetic patients with impaired renal function (Fig. 3). Five hours after iohexol injection, all healthy subjects had iohexol concentrations less than 40 μg/mL, while the patients showed higher levels (94.5–546.7 μg/mL). The observed iohexol concentrations after 12 h were 41.3–541.3 μg/mL in patients. No chromatographic interference of endogenous substances or other drugs was noted in the clinical samples.

Table 4
Stability of iohexol in serum and stock solution

Test conditions	Added concentration (μg/mL)	Number of observations	Measured concentration (μg/mL)	Bias (%)
Stability in serum				
Short-term (24 h, room temperature)	10	3	10.2	2.3
	100	3	94.4	5.6
	500	3	477.1	4.6
Long-term (2 months, −20 °C)	10	3	10.5	5.4
	100	3	99.9	1.1
	500	3	499.0	0.2
Freeze–thaw stability in serum (three cycles)				
	10	3	10.5	5.4
	100	3	99.9	0.1
	500	3	499.0	0.2
Post-preparative stability (in autosampler)				
	500	3	486.5	2.7
Stock solution stability	1500	3	1489.5	0.7

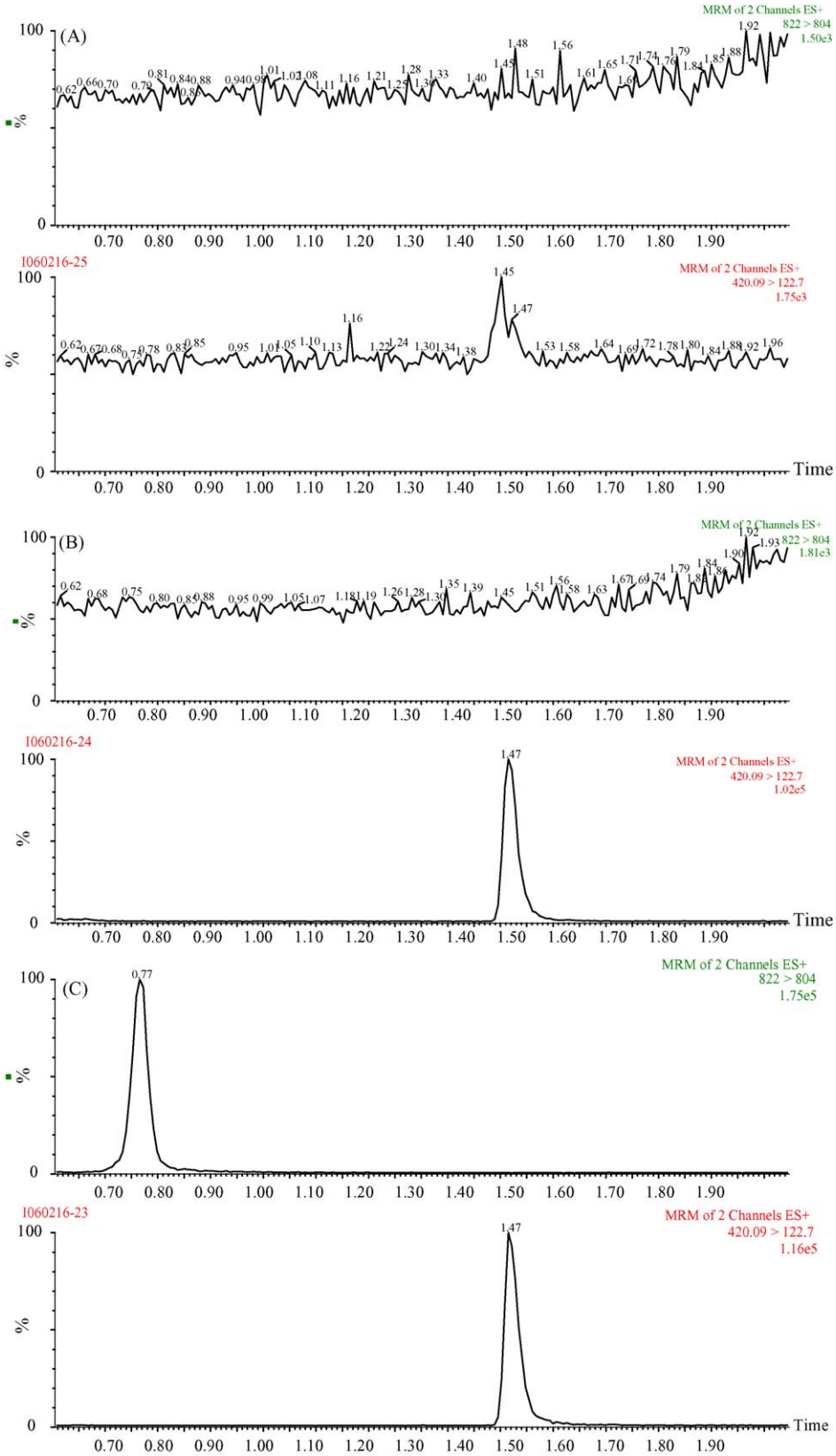

Fig. 1. Chromatograms of (A) pooled blank serum, (B) serum spiked with 10 μg/L of I.S. and (C) serum spiked with iohexol (250 μg/mL) and I.S. (10 μg/L) after protein precipitation.

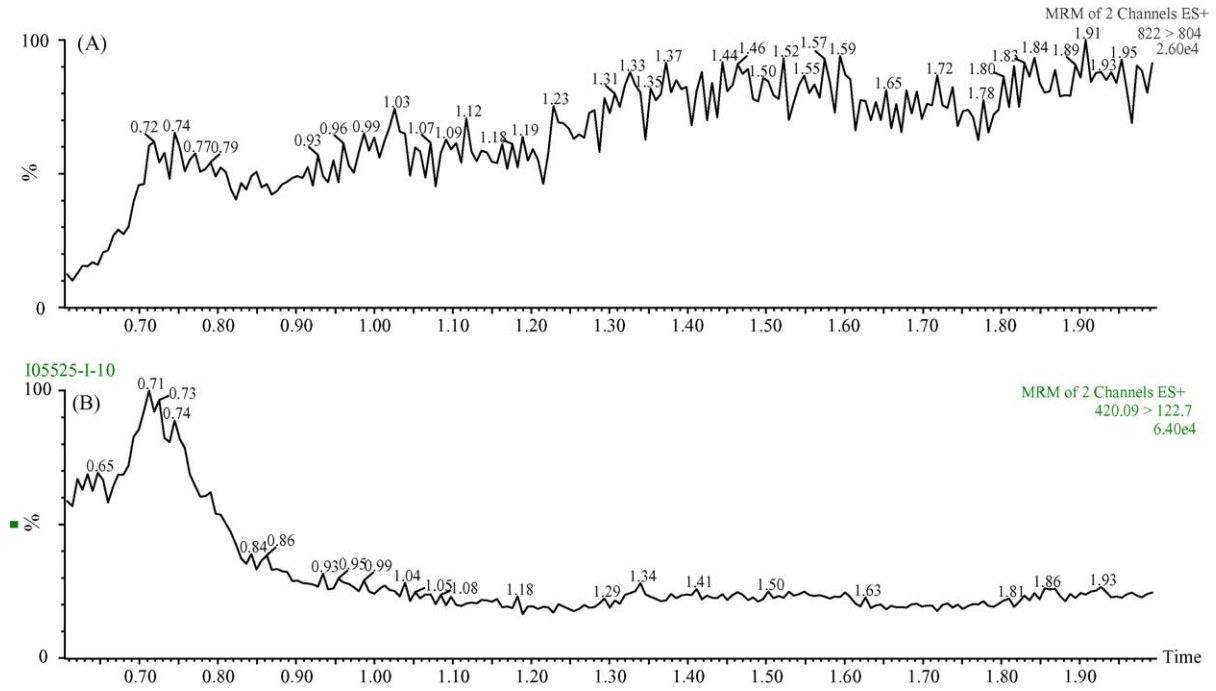

Fig. 2. Evaluation of ion suppression effect using pooled blank serum, injected in the LC/MS/MS system after protein precipitation, in parallel with continuous post-column infusion of Iohexol (100 ng/mL) (A) and I.S. (100 ng/mL) (B) in the electrospray source.

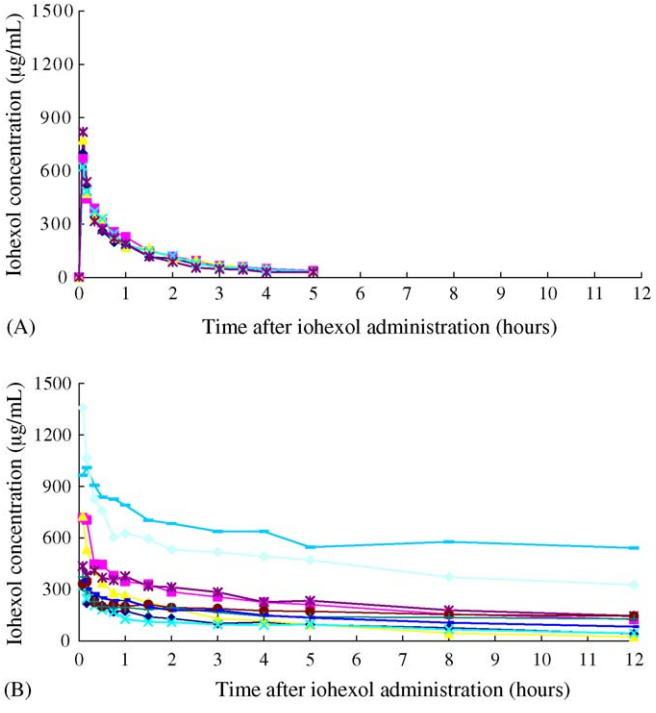

Fig. 3. Concentration–time profile of iohexol from (A) 5 healthy subjects and (B) 10 diabetic patients with chronic renal failure.

4. Discussion and conclusion

Accurate determination of GFR is very important for monitoring the progression of renal diseases, selecting the proper therapeutic options, and predicting the response to therapy. The gold standard for the measure of GFR uses inulin and other exogenously administered radioactive markers such as ^{51}Cr-EDTA which can also provide an indirect measure of GFR. However, due to the cost and complexity of the current tests for GFR, their utility in routine clinical practice is hindered. Serum creatinine and creatinine clearance are commonly used surrogate markers for renal function, but have several well-known limitations. Gaspari et al. [12] compared estimated GFR derived from 12 different equations including Cockroft and Gault formula, Jelliffe formula, and abbreviated MDRD by plasma iohexol clearance as reference method, and concluded that indirect estimates of GFR by prediction equations should be used with caution [12].

Demands for reliable and simple methods to perform measurements of GFR are increasing. Iohexol clearance testing using simple sampling strategy is performed more frequently in routine clinical setting. Our HPLC-MS/MS method for iohexol determination showed excellent analytical performance on measures of precision, accuracy, extraction recovery, sensitivity, stability, and specificity. The determination of iohexol clearance by HPLC-MS/MS provides several advantages when compared to HPLC methods. A major advantage over previously reported HPLC methods [1,8] is that the sample run time has been reduced to less than 3 min (retention times of iohexol was 0.7 min), which allows a faster turnaround time and high throughput. Whereas, the analytical times required in the previous HPLC methods were more than 10 min. Also, our HPLC-MS/MS method requires minimal sample volume (10 μL of serum), whereas HPLC methods often require more than 1 mL. The lower limit of quantification our method was 0.5 μg/mL, which is 20 times more sensitive than that of the recently reported HPLC method [8]. Our simple sample preparation method takes less than 5 min and provides clean chromatograms. There were no interfering peaks present in the clinical samples reviewed. As a variety of endogenous

or exogenous interfering substances in the patients' samples may cause significant errors in HPLC methods, LC–MS/MS method offering a far higher specificity has the advantage in the clinical setting. We could also produce excellent accuracy, extraction recovery, intra- and inter-day precision over a wide analytical range. The linear assay range (0.5–1500 μg/mL) covered all concentrations observed in 210 clinical samples (24.2–1358.36 μg/mL) from the 15 subjects with a variety of kidney function. In the previous studies, the injection volumes of iohexol were 5 mL [1], 10 mL [8], and 20 mL [2,3], and the blood sampling volume was 10 mL at every time point [2,8]. The observed iohexol concentrations were high enough for HPLC-UV analysis at a dosage given according to the testing protocol, originally developed for HPLC-UV method. We injected 5–9 mL of iohexol in each subject according to the body weight and collected less than 3 mL of EDTA blood. Considering the excellent sensitivity and minimal sampling requirement of our analytical method, the amount of iohexol can be reduced to far less than 5 mL and less than 1 mL of blood collection may be sufficient to determine plasma iohexol concentration. Our method can preclude unnecessary large volumes of bolus IV injection of iohexol or blood collection, which is really advantageous in the clinical practice, especially for the patients with renal dysfunction. HPLC methods are generally laborious, time-consuming, less sensitive, and have limited specificity. Despite the high cost of the instrument, LC–MS/MS is being implemented in more and more laboratories. Accordingly, simple, rapid, and accurate LC–MS/MS methods have replaced existing HPLC methods for many analytes.

In conclusion, our new technique for iohexol measurement using HPLC-MS/MS provides a rapid, simple, and reliable method for the determination of GFR. With its high speed, simplicity, specificity, and minimal sample requirements, our method can be readily incorporated into routine operations in the clinical setting.

References

[1] E. Krutzen, S.E. Back, I. Nilsson-Ehle, P. Nilsson-Ehle, J. Lab. Clin. Med. 104 (1984) 955.
[2] E. Brandstrom, A. Grzegorczyk, L. Jacobsson, P. Friberg, A. Lindahl, M. Aurell, Nephrol. Dial. Transplant 13 (1998) 1176.
[3] H.G. Lindblad, U.B. Berg, Acta Paediatr. 83 (1994) 418.
[4] S.C. Brown, P.H. O'Reilly, J. Urol. 146 (1991) 675.
[5] T.A. Chowdhury, P.H. Dyer, W.A. Bartlett, E.S. Legge, S.M. Durbin, A.H. Barnett, S.C. Bain, Clin. Chim. Acta 277 (1998) 153.
[6] M.V. Rocco, V.M. Buckalew Jr., L.C. Moore, Z.K. Shihabi, Am. J. Nephrol. 16 (1996) 138.
[7] P. Olofsson, E. Krutzen, P. Nilsson-Ehle, Eur. J. Obstet. Gynecol. Reprod. Biol. 64 (1996) 63.
[8] R.S. Soman, H. Zahir, F. Akhlaghi, J. Chromatogr. B 816 (2005) 339.
[9] S.E. Back, P. Masson, P. Nilsson-Ehle, Scand. J. Clin. Lab. Invest. 48 (1988) 825.
[10] M.V. Rocco, V.M. Buckalew Jr., L.C. Moore, Z.K. Shihabi, Am. J. Kidney Dis. 28 (1996) 173.
[11] W.E. Braselton, K.J. Stuart, J.M. Kruger, Clin. Chem. 43 (1997) 1429.
[12] F. Gaspari, S. Ferrari, N. Stucchi, E. Centemeri, F. Carrara, M. Pellegrino, G. Gherardi, E. Gotti, G. Segoloni, M. Salvadori, P. Rigotti, U. Valente, D. Donati, S. Sandrini, V. Sparacino, G. Remuzzi, N. Perico, Am. J. Transplant 4 (2004) 1826.