Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 861 (2008) 95–100
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

A highly sensitive assay for ritodrine in human serum by hydrophilic interaction chromatography-tandem mass spectrometry

Tomofumi Ohmori [a], Mitsuhiro Nakamura [b,*], Shin Tada [c], Tadashi Sugiyama [d], Yoshinori Itoh [e], Yasuhiro Udagawa [c], Kazuyuki Hirano [a]

[a] *Laboratory of Pharmaceutics, Gifu Pharmaceutical University, 5-6-1, Mitahora-higashi, Gifu 502-8585, Japan*
[b] *Laboratory of Drug Informatics, Gifu Pharmaceutical University, 5-6-1, Mitahora-higashi, Gifu 502-8585, Japan*
[c] *Department of Obstetrics and Gynecology, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aischi 470-1192, Japan*
[d] *Laboratory of Pharmacy Practice and Social Science, Gifu Pharmaceutical University, 5-6-1, Mitahora-higashi, Gifu 502-8585, Japan*
[e] *Department of Pharmacy, Gifu University Hospital, 1-1 Yanagido, Gifu 501-1194, Japan*

Received 1 June 2007; accepted 7 November 2007
Available online 19 November 2007

Abstract

We developed a sensitive assay for ritodrine (RTD), a β_2-adrenergic agonist, in human serum. This method was based upon the selective and sensitive technique by a tandem mass spectrometry (MS/MS) using a hydrophilic interaction chromatography (HILIC) technique. This method involved a mixed-mode cation-exchange solid-phase extraction of RTD and isoxsuprine, the internal standard (IS), from serum with Waters Oasis® MCX cartridges. The detection was made using a Micromass Quattromicro™ API LC–MS/MS system with electrospray ionization source in positive ion mode. The separation of the analytes was achieved within 4 min on a silica column with a mobile phase of ammonium acetate (10 mM, pH 4.5) and acetonitrile (10:90, v/v). Multiple reaction monitoring was utilized by monitoring 288.2 → 121.1 for RTD, 302.2 → 107.0 for IS. The calibration curve for RTD was linear over a range of 0.5–1000 ng/mL. When 50 μL serum was used for extraction, the lower limit of quantification was 0.39 ng/mL (97.5 fg on-column). The percent coefficient of validation for accuracy and precision (inter- and intra-day) was less than 9.8% and the recovery was ranged from 83.5 to 94.7% for RTD. This method enabled us to successfully determine RTD in maternal and fetal sera.

Keywords: Ritodrine; Tandem mass spectrometry; Hydrophilic interaction chromatography; Solid-phase extraction

1. Introduction

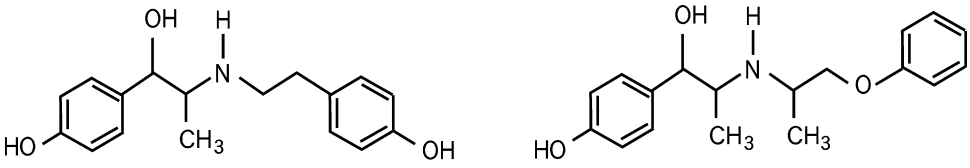
Ritodrine hydrochloride, 4-[2-[2-hydroxy-2-(4-hydroxyphenyl)-1-methyl-ethyl] aminoethyl] phenol (RTD, Fig. 1), is a β_2-adrenergic agonist with a potent dilatory action on uterine smooth muscles [1–4], and thus used clinically for the management of preterm labor. However, the compound possesses cardiovasucular and metabolic side effects due to the stimulation of β_1-adrenoceptors at relatively high concentrations [1–4]. Thus, the monitoring of serum concentration of RTD would be needed to avoid these adverse effects.

There have been several reports on the assay for RTD in biological fluids using a reversed-phase (RP) high-performance liquid chromatography (HPLC) with electrochemical [5–7] or fluorescence detection [8]. However, most of those methods are not suited to the pharmacokinetic analysis on RTD in fetus or neonates because of the requirement of large volume of plasma specimens (more than 1 mL) for quantitation. Furthermore, the extraction procedures such as liquid–liquid extraction are time-consuming in most cases [5–8]. At present, solid-phase extraction (SPE) is the most common extraction method. Josefsson and Sabanovic [9] have reported that the Oasis® MCX, a cation-exchange solvent, is highly suitable for the preparation of some β agonists that are structurally related amino alcohols. Therefore, we considered it possible to use Oasis® MCX for the separation of RTD from serum samples.

Recently, several chromatographic methods with mass spectrometry (MS) have been recognized as a powerful tool for the determination of RTD [10,11]. Fesser et al. [11] have reported a LC-tandem mass spectrometry (MS/MS) method for the

* Corresponding author. Tel.: +81 58 237 3931; fax: +81 58 237 5979.
E-mail address: mnakamura@gifu-pu.ac.jp (M. Nakamura).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.11.010



1) Ritodrine

2) Isoxsuprine
(Internal Standard; IS)

Fig. 1. Chemical structures of ritodrine and isoxsuprine.

determination of RTD in bovine liver and retina, using a RP column. Since RTD is a hydrophilic compound, the retention of this compound on RP columns is largely restricted. In order to separate the analyte from the matrix interference, the use of organic solvent in the mobile phase for RP-HPLC should be limited as possible. However, the low organic content is not optimal for electrospray mass spectrometric detection, since it reduces the sensitivity of MS. Recently, hydrophilic interaction chromatography (HILIC) coupled with MS has been reported for the analysis of polar molecules in biological matrix [12–14].

The aim of the present study was to develop a HILIC-MS/MS method that was simple and sensitive enough to determine the small amount of RTD in human fetal serum.

2. Experimental

2.1. Chemicals and materials

RTD and isoxsuprine (IS), an internal standard, were obtained from Kissei Pharmaceutical Co. Ltd. (Nagano, Japan) and Daiichi Pharmaceutical Co. Ltd. (Osaka, Japan), respectively. β-Glucuronidase of type H-2 from Helix Pomatia was purchase from Sigma Chemical Co., St. Louis, Mo, USA [G-0876]). Methanol and acetonitrile of HPLC grade were purchased from Kishida Chemical (Osaka, Japan). Purified water obtained through Milli-Q system (Millipore, Bedford, MA, USA) was used throughout the experiment. All reagents were of analytical-reagent grade.

2.2. Standard solutions

Primary stock solutions of RTD (100 μg/mL) and IS (10 μg/mL) were separately prepared in distilled water, respectively. Working solutions for calibration and controls were prepared by appropriate dilution in water. RTD and IS solutions were stored at 4 °C. Working standard solutions (100 μL) were added to 4900 μL drug-free human serum to obtain RTD concentrations of 0.5, 1, 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL. Quality control (QC) samples at 0.5, 5, 50, 100 and 500 ng/mL were prepared by adding 100 μg/mL of the appropriate working standard solutions to drug-free human serum (10 mL). The resultant serum standards and quality control standards were pipetted into 15 mL polypropylene tubes and stored at −30 °C until analysis.

2.3. Serum specimens of patients

The serum samples were obtained from patients at Fujita Health University Hospital. The written informed consent was obtained from nine pregnant women during preterm labor who received intravenous RTD. Maternal blood samples were collected both at the discontinuation of RTD infusion and at delivery. Umbilical blood samples were collected immediately after delivery.

2.4. Sample preparation

The solid-phase extraction method was conducted. A 50 μL aliquot of serum samples was mixed with 20 μL of IS solution (1 μg/mL) and 30 μL of formic acid (2% v/v), and vortex-mixed for 20 s. The samples were then loaded onto an Oasis® MCX cartridge (30 mg) (Waters Co., Milford, USA) pre-conditioned with methanol (1 mL) and followed by 2% formic acid (1 mL). The cartridge was rinsed with 2% formic acid (1 mL) followed by methanol (1 mL), and then eluted with 2% ammonia in methanol (1 mL). Then, the eluent volume of 5 μL was injected into the LC–MS/MS system.

2.5. RTD conjugate hydrolysis

To examine whether conjugated metabolites of RTD were converted to the parent compound during extraction, the maternal serum and the umbilical cord serum of patients receiving the RTD infusions were split into aliquots of 60 μL. The hydrolysis procedure employed in the present study was originally employed by Brashear et al. [15]. β-Glucuronidase with both glucuronidase and sulfatase activities was used for the enzymatic hydrolysis of the conjugated RTD, which gave the total amount of conjugated RTD, the sum of the glucuronide and sulfate conjugates[15]. One aliquot was extracted, as described above, and intact RTD measured. To another aliquot was added 60 μL of 0.2 M sodium acetate buffer (pH 5) and sufficient β-glucuronidase activity of 2500 U/mL. Aliquot were incubated for 20 h at 37 °C. After incubation, the liberated RTD was immediately quantitated by the method described previously.

2.6. RTD and RTD conjugate stability during extraction

It is known that glucuronide conjugates may be hydrolyzed by strong acid and heat. We did not expose serum specimens to high

concentrations of HCl or high temperatures during the extraction process. QC samples and patients' sera were loaded and left on Oasis® MCX cartridge for 120 min at ambient temperature and then taken the through solid-phase extraction purification, and the measured concentrations of RTD for the patient samples were compared with the same sera that had been extracted immediately.

2.7. HILIC-MS/MS methods

The HILIC-MS/MS analysis was performed by using a Waters 2695 HPLC system (Milford, MA, USA) coupled with a Micromass Quattromicro™ API triple-quadrapole mass spectrometer (Manchester, UK). The mass spectrometer was operated using an electrospray atmospheric pressure ionization (ESI) source in positive ion mode with multiple reaction monitoring (MRM). The separation was performed on a Unison™ UK-Silica (3 μm porous silica, 2.0 mm i.d. × 50 mm, Imtakt, Kyoto, Japan) using a mixture of ammonium acetate (10 mM, pH 4.5) and acetonitorile (10:90, v/v) at a flow-rate of 0.4 mL/min. The column and autosampler tray temperatures were stabilized at 30 °C and 4 °C, respectively. A 5 μL volume of eluate was injected and the LC effluent was directed to the ESI source without splitting. The analytical run time was 4 min. Sensitivity of MRM was optimized by infusing a mixture of RTD and IS, 1 ng/mL each, in the mobile phase. The capillary voltage was maintained at 3.5 kV. The cone voltages were set to 24 V for RTD and 21 V for IS. The extractor voltage was set to 3 V. Nebulizing gas and electrospray source temperatures were stabilized at 400 and 120 °C, respectively. Ions were activated at 26 eV of collision energy for RTD and 30 eV for IS, and the indicated argon pressure was set at 3.0×10^{-3} Torr. Detection of the ions was performed by monitoring the transitions of *m/z* 288.2 to 121.1 for RTD and *m/z* 302.2 to 107.0 for IS. The dwell time for each transition was 400 ms and the interchannel delay was 20 ms. Peak areas for all components were automatically integrated using Masslynx™ NT 4.0 software (Micromass, Manchester, UK).

2.8. Validation of the HILIC-MS/MS methods

Validation samples were prepared and analyzed to evaluate the intra-day and inter-day accuracy and precision of the analytical method in human serum. Five replicates of each of the validation concentration serum (0.5, 5, 50 and 500 ng/mL) were analyzed along with one set of standard samples on each of 5 days using the same instrument. The limit of detection (LOD) was determined from signal to noise ratio (S/N) of 3 and the lower limit of quantification (LLOQ) from S/N of 10.

The extraction efficiency of RTD and IS were determined by comparing the peak areas of extracted QC samples to the peak areas of extracted solutions spiked with standard and IS. In addition, the effect of the matrix on the detection of the analytes (matrix suppression) was evaluated by comparing the extracted blanks spiked with standard and internal standard to neat standards at the same concentrations. The selectivity of the assay was investigated by processing and analyzing blanks prepared from six independent lots of control serum; the blanks surveyed for interfering peaks at the transition monitored.

The stability of RTD in serum was tested at two concentrations (5 and 100 ng/mL as low and high value) with six determinations for each after three freeze/thaw cycles at −30 °C. Post-preparative stability was assessed on the autosampler for 3, 6 and 24 h. The long-term stability test was performed by analytes stored at −30 °C for 31 days.

2.9. Application

The HILIC-MS/MS method was used for the determination of RTD in the serum and umbilical cord serum of patients.

3. Results and discussion

3.1. HILIC-MS/MS

The electrospray ionization of RTD and IS produced the $[M+H]^+$ ions at 288 and 302, respectively, under positive-ionization conditions. The product ion spectra at *m/z* 121 and *m/z* 107 were produced as the prominent product ions for RTD and IS, respectively. The quantitative analysis was performed using the MRM mode due to the high selectivity and sensitivity of MRM data acquisitions: *m/z* 288.2 → 121.1 for RTD and *m/z* 302.2 → 107.0 for IS. Desolvation temperature, collision energy and argon gas flow were determined when observing maximum response of the product ion. Furthermore, following mobile phases were tested: aqueous ammonium acetate (10 mM, pH 4.5)/acetonitrile (10:90), ammonium formate (10 mM, pH 3.1)/acetonitrile (10:90), aqueous ammonium acetate (10 mM, pH 4.5)/methanol (10:90). The best result in terms of peak intensity and peak shape was obtained using aqueous ammonium acetate (10 mM, pH 4.5)/acetonitrile (10:90).

HILIC methods operated with the silica column and low aqueous-high organic mobile phase have been proved to be suitable for the analysis of polar compounds in biological fluids [12–14]. The higher organic content in mobile phase for HILIC resulted in the improvement of sensitivity compared to RP-HPLC. The sensitivity of the RTD assay is reported to be higher in HILIC-MS/MS method than in RPLC-MSMS, which enabled to reduce the required sample volume (50 μL in the present study).

3.2. Method validation

Fig. 2 shows the representative MRM chromatograms obtained from blank human serum, human serum spiked with RTD. The retention times of RTD and IS were 2.8 and 1.3 min, respectively. No appreciable interfering peaks were detected in human serum. Sample carryover effect was not observed. In order to demonstrate matrix lot-to-lot reproducibility, six different blank serums were spiked with the RTD at LLOQ, 0.39 ng/mL. The coefficient of variation (C.V.) varied from 7.0 to 9.0% and the recoveries ranged from 94.3 to 102.8% (Table 1). This result indicated that there was no significant lot-to-lot variation in matrix effect.

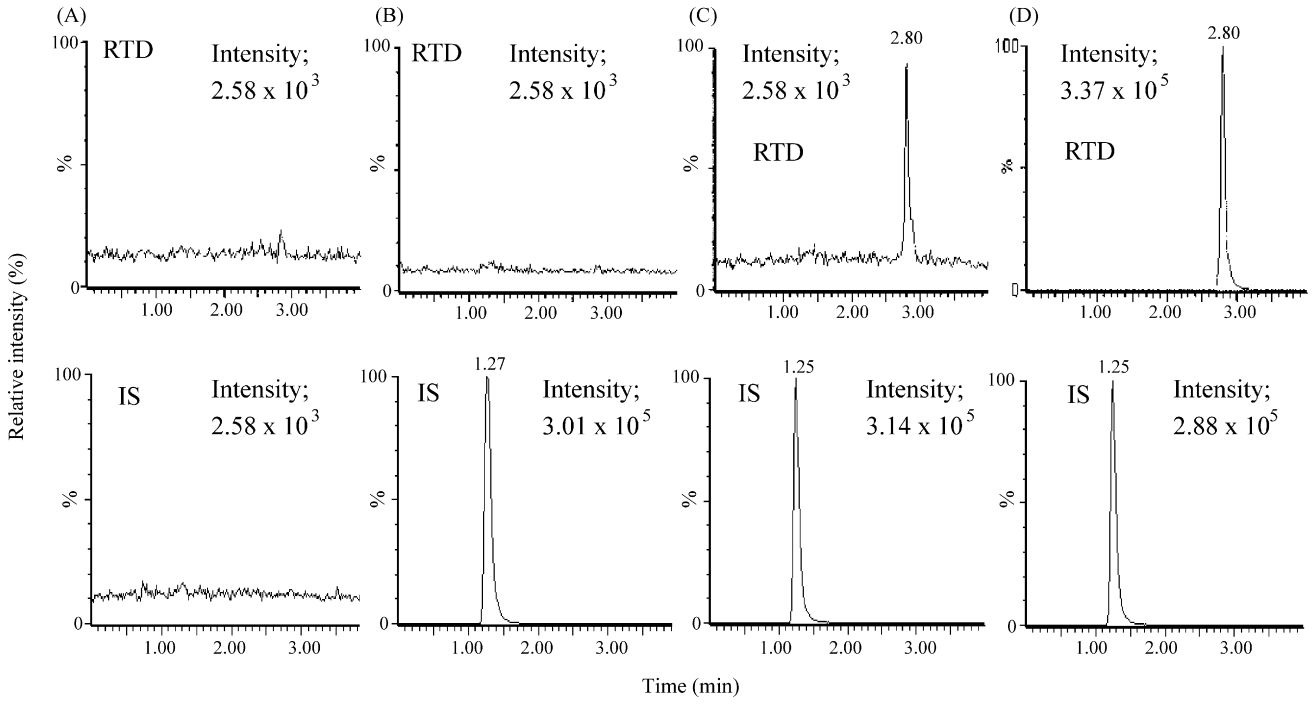


Fig. 2. MRM chromatograms obtained from (A) blank human serum; (B) human serum spiked with IS; (C) human serum spiked with 0.5 ng/mL RTD and IS; (D) human serum spiked with 100 ng/mL RTD and IS.

The linear regression of the peak-area ratios versus concentrations was fitted over the concentration range of 0.5–1000 ng/mL in human serum. The calibration curve fitted with the following equation:

$$y = 0.006x + 0.023$$

where y is the peak-area ratios of RTD to IS, and x is the concentration (ng/mL) of RTD. The correlation coefficient (r) was 1.000 ($n = 3$).

LOD and LLOQ in our method were 0.12 (30.0 fg on column) and 0.39 ng/mL (97.5 fg on-column) using 50 μL serum, respectively. Precision and accuracy of LLOQ was within 10%. Reported LOD values are approximately 0.6 ng/mL (60 pg on-column), using 1 mL plasma [5,7]. In other report, LOD during on-column injection were found to be 0.2 ng [6] and 0.67 ng [8], using 1 mL of plasma and blood, respectively. Fesser et al. [11] have reported that their LOD was 0.2 ng/g (RTD was extracted from 10 g liver). Due to the need for a large amount of biological sample, these methods are not suitable for quantifying RTD in fetal or neonatal serum. In contrast, the sensitivity of our method was highly improved compared with the conventional LC methods. Thus, as low as 50 μL aliquot of serum specimens was enough to determine RTD.

Table 1
Matrix lot-to-lot reproducibility ($n = 3$) at LLOQ (0.39 ng/mL)

Matrix lot	Mean	C.V. (%)	Recovery (%)
1	0.39	8.3	100.0
2	0.37	9.0	95.9
3	0.40	7.2	102.8
4	0.40	7.9	102.5
5	0.40	6.7	101.6
6	0.37	7.0	94.3

Table 2 summarizes the intra- and inter-day precision and accuracy for RTD from QC samples. In this assay, the intra- and inter-day precision ranged from 2.7 to 3.7% and 5.1 to 9.8% for each QC levels, respectively. The mean accuracy values ranged between 97.6 and 109.0%. The detection sensitivity was high enough to determine RTD in serum of patients who received the compound. The replicate analysis indicated low C.V. Both accuracy and precision in the determination of RTD were satisfactory.

Oasis® MCX contains a mixed-mode solvent with both reversed-phase and strong cation-exchange functionalities [9]. The retention of RTD on Oasis® MCX was demonstrated in acidic conditions when the amines are in their protonated ionized state. The recoveries of RTD spiked in serum at four concentrations were in the range of 83.5–94.7% (Table 3). Several extraction protocols such as SPE, Oasis HLB and Sep-Pak C18 were tested, but Oasis® MCX was found to be most reproducible and showed the highest recovery between them.

Table 4 shows the stability of RTD during sample handling (three freeze/thaw, post-preparation stability) and long-term stability. Post-preparation storage before analysis hardly influenced the quantification of RTD. Solutions of RTD and the IS appear stable in the autosampler for at least 6 h without any significant loss.

RTD concentrations of QC sample (spiked only RTD) and patients' sera (contain both RTD and its conjugates) were stable for 120 min during the extraction procedure used for the assay (data not shown). Furthermore, we have carefully exam-

Table 2
The intra-, inter-day precision and accuracy for the measurement of RTD in human serum ($n = 5$)

Nominal concentration (ng/mL)	Intra-day			Inter-day		
	Mean ± S.D.	Precision (%)	Accuracy (%)	Mean ± S.D.	Precision (%)	Accuracy (%)
0.5	0.55 ± 0.02	3.7	109.0	0.50 ± 0.05	9.8	100.4
5	5.3 ± 0.1	2.7	105.6	5.1 ± 0.3	5.1	101.7
50	52.6 ± 1.7	3.2	105.3	48.8 ± 2.8	5.8	97.6
500	505.7 ± 18.2	3.6	101.1	507.0 ± 33.5	6.6	101.4

Table 3
Recoveries of RTD from human serum ($n = 5$)

Nominal concentrations (ng/mL)	Mean ± S.D. (%)	C.V. (%)
0.5	90.0 ± 7.9	8.7
5	83.5 ± 3.0	3.6
50	90.2 ± 4.9	5.5
500	94.7 ± 4.3	4.6

ined the separation of RTD and conjugated RTD on solid-phase extraction procedure using Oasis® MCX. Using two patients' sera (RTD concentration; 0.5 and 15.6 ng/mL), conjugated RTD, which was determined as liberated RTD after β-glucuronidase treatment, was not eluted in 2% formic acid fraction at all. Most conjugated RTD was observed in methanol fraction in the washing step. On the contrary, over 90.0% of intact RTD were observed in methanol containing 2% ammonia in the elution step. Therefore, we think that conjugated RTD exerts, if any, a minimal effect on intact RTD determination. Because of the lack of authentic RTD glucuronide, we could not evaluate in-source fragmentation and chromatographic separation in detail.

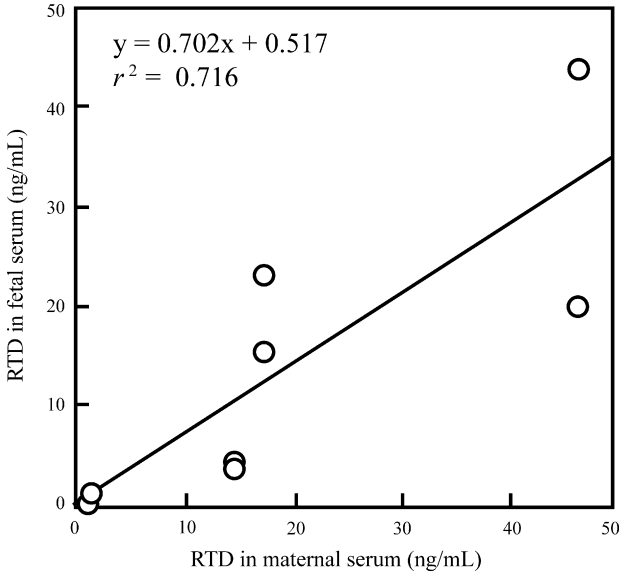


Fig. 3. Relationship between RTD concentrations in maternal serum and those in fetal serum.

3.3. Application

The present HILIC-MS/MS method for a rapid and sensitive determination of RTD was found to be applicable to the assay of RTD in maternal and fetal sera.

RTD is inactivated in humans by sulfate and glucuronide conjugation and excreted in the urine [16,17]. The conjugated form of RTD in the maternal serum and the umbilical cord serum ranged from 87.0 ± 3.8 and 89.6 ± 4.7%, respectively. These

Table 4
Stability of RTD in human serum

Nominal concentration (ng/mL)	Mean ± S.D. (ng/mL)	C.V. (%)	Accuracy (%)
Three freeze/thaw cycles ($n = 6$)			
5	5.1 ± 0.1	1.8	102.9
100	101.3 ± 5.3	5.2	101.3
Post-preparation stability ($n = 6$)			
After 3 h			
5	4.6 ± 0.1	1.8	91.6
100	93.8 ± 0.4	0.5	93.8
After 6 h			
5	4.4 ± 0.1	1.8	88.9
100	93.9 ± 1.8	1.9	93.9
After 24 h			
5	3.7 ± 0.1	3.0	73.2
100	81.8 ± 3.0	3.7	81.8
Stability for 31 days at below −30 °C ($n = 6$)			
5	4.6 ± 0.2	5.1	92.4
100	101.0 ± 1.6	1.6	101.1

values are comparable to those reported by other investigators [16,17].

Fig. 3 shows a good correlation of RTD concentrations in maternal and fetal sera. The regression equation for this correlation was as follows:

$$y = 0.702x + 0.517 (r^2 = 0.716)$$

where y is the RTD concentration (ng/mL) of fetal serum, x is the concentration (ng/mL) of maternal serum and r is the correlation coefficient.

Several adverse effects such as maternal and fetal tachycardia and maternal hypertension have been reported to occur after intravenous injection of RTD [18–20]. However, little is known about the relationship between RTD concentrations and such adverse effects. Investigations on this issue are currently being pursued.

4. Conclusion

We developed a rapid and sensitive HILIC-MS/MS method for the analysis of RTD in human serum. This method was highly sensitive (LLOQ 0.39 ng/mL, 97.5 fg on-column) requiring 50 μL volume of samples, and thus successfully applied to determine RTD in the maternal and fetal sera.

References

[1] T.P. Barden, Am. J. Obstet. Gynecol. 112 (1972) 645.
[2] D.J. Nochimson, H.D. Riffel, S.Y. Yeh, M.S. Kreitzer, R.H. Paul, E.H. Hon, Am. J. Obstet. Gynecol. 118 (1974) 523.
[3] A.S. Siimes, R.K. Creasy, Am. J. Obstet. Gynecol. 126 (1976) 1003.
[4] The Canadian Preterm Labor Investigators Group, N. Engl. J. Med. 327 (1992) 308.
[5] P. Kuhnert, P. Erhard, A. Dixon, B. Kuhnert, T. Gross, J. Liq. Chromatogr. 6 (1983) 2775.
[6] L.S. Lin, S.N. Caritis, L.K. Wong, J. Pharm. Sci. 73 (1984) 131.
[7] T.L. Gross, B.R. Kuhnert, P.M. Kuhnert, M.G. Rosen, N.J. Kazzi, Obstet. Gynecol. 65 (1985) 793.
[8] A.S. Gross, K.F. Brown, J.A. Baird-Lambert, R.L. Nation, J. Chromatogr. 416 (1987) 400.
[9] M. Josefsson, A. Sabanovic, J. Chromatogr. A 1120 (2006) 1.
[10] W.T. Brashear, B.R. Kuhnert, R. Wei, Drug Metab. Dispos. 18 (1990) 488.
[11] A.C. Fesser, L.C. Dickson, J.D. MacNeil, J.R. Patterson, S. Lee, R. Gedir, J. AOAC. Int. 88 (2005) 61.
[12] R.N. Xu, L. Fan, M.J. Rieser, T.A. El-Shourbagy, J. Pharm. Biomed. Anal. 44 (2007) 342.
[13] Q. Song, W. Naidong, J. Chromatogr. B 830 (2006) 135.
[14] T.M. Baughman, W.L. Wright, K.A. Hutton, J. Chromatogr. B 852 (2007) 505.
[15] W.T. Brashear, B.R. Kuhnert, R. Wei, Clin. Pharmacol. Ther. 43 (1988) 634.
[16] M.R. Wright, D.W. Rurak, M.P. van der Weyde, S.M. Taylor, J.E. Axelson, J. Pharmacol. Exp. Ther. 258 (1991) 897.
[17] E. Hayashi, Y. Kishimoto, K. Tada, T. Kudo, K. Sekiba, Acta Med. Okayama 46 (1992) 443.
[18] R. Gandar, L.W. de Zoeten, J.B. van der Schoot, Eur. J. Clin. Pharmacol. 17 (1980) 117.
[19] M. van Lierde, K. Thomas, J. Perinat. Med. 10 (1982) 119.
[20] C.A. Holleboom, J.M. Merkus, L.W. van Elferen, M.J. Keirse, Br. J. Obstet. Gynaecol. 100 (1993) 1107.