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Short Communication

A validated liquid chromatography tandem mass spectrometry method for simultaneous determination of pioglitazone, hydroxypioglitazone, and ketopioglitazone in human plasma and its application to a clinical study

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ABSTRACT

A simple and rapid high performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed for the simultaneous determination of pioglitazone and its active metabolites hydroxypioglitazone and ketopioglitazone in human plasma. Samples were processed by protein precipitation with acetonitrile and selective phospholipid depletion in a 96-well plate format. The method used deuterated internal standards for each analyte. Chromatographic separation was achieved with gradient elution on a Hypersil GOLD C18 column. The mass spectrometer was operated in electrospray positive ion mode with detection by selected reaction monitoring using the transitions *m/z* 357.1 > 134.0 for pioglitazone, *m/z* 373.1 > 150.0 for hydroxypioglitazone, and *m/z* 371.0 > 148.0 for ketopioglitazone. A linear standard curve was established for the range of 10–1800 ng/mL for all three analytes. Intra-run and inter-run precision and accuracy (relative error) were less than 15%, and the mean extraction recoveries of all analytes were more than 87.8%. The validated method is sensitive and selective and was successfully applied to analyze clinical samples obtained from patients with nonalcoholic fatty liver disease taking pioglitazone.

1. Introduction

Pioglitazone is a thiazolidinedione that acts as an insulin sensitizer; it is indicated for the management of type 2 diabetes mellitus [1]. In addition to improvements in insulin sensitivity, glucose control, and glucose tolerance, pioglitazone is also reported to have beneficial effects in other diseases, such as nonalcoholic steatohepatitis [2]. Pioglitazone is given orally at a starting dose of 15 mg daily and titrated to effect, up to 45 mg daily [1,3]. In humans, orally administered pioglitazone is rapidly and well absorbed with a peak serum concentration achieved in about 1.5 h (range 0.5–3 h) and bioavailability of approximately 83% [4,5]. Pioglitazone is extensively metabolized in the liver by cytochrome P450 (CYP) enzymes, primarily CYP2C8, before renal and fecal elimination [4,5]. Hydroxypioglitazone (often referred to as M-IV) and ketopioglitazone (M-III) are the main active metabolites formed via CYP-mediated metabolism (Fig. 1A) [4,6,7]. The mean terminal elimination half-life of pioglitazone after intravenous administration is 5.8 h (range 3.5–9 h), while the half-lives of hydroxypioglitazone and ketopioglitazone are much longer at 26–28 h [4,5]. Because pioglitazone is widely used clinically, and may also be used as a probe drug to measure *in vivo* CYP2C8 enzymatic activity [7], a sensitive and selective assay for pioglitazone and its active metabolites is needed.

Several methods have been reported to qualitatively or quantitatively detect pioglitazone with or without the metabolites. Pioglitazone and metabolite concentrations in human plasma and urine have been determined by liquid chromatography (LC) with ultraviolet (UV) detection after solid-phase or liquid–liquid extraction [8–10]. UV–visible spectrophotometry was also used to measure concentrations of pioglitazone and metformin together [11]. More recently, LC with tandem mass spectrometry

Abbreviations: CYP, cytochrome P450; ESI, electrospray ionization; LC, liquid chromatography; LLOQ, lower limit of quantitation; MS/MS, tandem mass spectrometry; QC, quality control; RE, relative error; RSD, relative standard deviation; SD, standard deviation; UV, ultraviolet.

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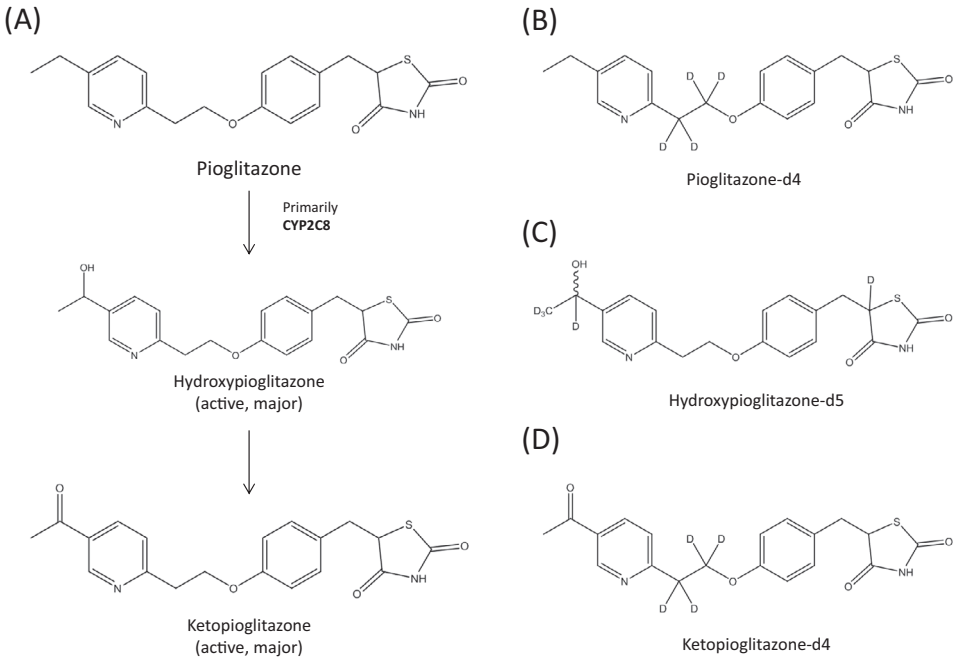

Fig. 1. (A) Pioglitazone metabolic pathway [4,6,7]. Hydroxypioglitazone and ketopioglitazone are the major active metabolite in humans, while other minor active (M-II) and inactive (M-I, M-V, M-IV) metabolites exist. Structures of deuterated internal standards are shown: (B) pioglitazone-d4, (C) hydroxypioglitazone-d5 and (D) ketopioglitazone-d4.

(LC–MS/MS) was used, after sample processing by liquid–liquid extraction, to measure concentrations of pioglitazone as well as other anti-diabetic agents [12]. Direct-injection LC–MS/MS has also been used to determine pioglitazone concentration [13]. Recently, an LC–MS/MS assay was developed to measure the concentrations of pioglitazone, hydroxypioglitazone, and metformin in human plasma after sample processing by protein precipitation in tubes [14]. Similarly, an LC–MS/MS method was used to measure the concentration of pioglitazone and other anti-diabetic agents, such as glipizide, glyburide, repaglinide, rosiglitazone, and nateglinide [15]. An assay of pioglitazone and flurbiprofen using LC with time-of-flight mass spectrometry has been reported to detect metabolites of each compound [16]. Hydroxypioglitazone and ketopioglitazone are pharmacologically active and present in significant amount in plasma [4,6,7]. Therefore, quantification of these metabolites is important when analyzing samples from clinical trials. However, many of the previous assays estimated only the concentration of parent pioglitazone and not both of the active metabolites. In addition, previously reported methods were relatively time-consuming due to involvement of multiple processing steps, such as liquid–liquid extraction.

Herein, we report a novel LC–MS/MS assay to determine pioglitazone, hydroxypioglitazone, and ketopioglitazone plasma concentrations in a single run. The method has certain advantages over previously reported methods including rapid sample processing based on protein precipitation, selective phospholipid depletion, and filtration in a 96-well plate format, utilization of deuterated internal standards, and small sample volume requirements (100 μL human plasma). The method is suitable for analyzing plasma samples from clinical trials, including pharmacokinetic and drug-drug interaction studies.

2. Materials and methods

2.1. Chemicals and reagents

Pioglitazone hydrochloride (98% purity), hydroxypioglitazone (M-IV, 97% purity), ketopioglitazone (M-III, 98% purity), pioglitazone-d4 (97% chemical and 96% isotopic purity), hydroxypioglitazone-d5 (98% chemical and 96% isotopic purity), and ketopioglitazone-d4 (98% chemical and >98% isotopic purity) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Chemical structures of the deuterated internal standards are shown in Fig. 1(B–D). HPLC grade acetonitrile and methanol and analytical grade formic acid were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Human EDTA plasma was obtained from the UF Health blood bank (Gainesville, FL, USA). The plasma was screened for the presence of pioglitazone, hydroxypioglitazone, and ketopioglitazone prior to use. HPLC grade deionized water from a Barnstead Nanopure Diamond UV Ultrapure Water System (Dubuque, IA, USA) was used.

2.2. Instrumentation and chromatographic conditions

The LC–MS/MS system consisted of a Surveyor HPLC autosampler, surveyor MS quaternary pump, and a TSQ Quantum Discovery triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA, USA). The TSQ quantum mass spectrometer was equipped with an electrospray ionization (ESI) source, which was positioned orthogonal to the ion transfer capillary tube. The temperature of the autosampler was maintained at 10 °C. The analytical column was a Hypersil GOLD, 50 mm × 2.0 mm, 5 μm, C18 column (Thermo Scientific, San Jose, CA, USA). The mobile phase was made of deionized water and acetonitrile, both of which contained 0.2% formic acid and 5 mM ammonium acetate. A gradient system was set to start with the ratio of deionized water:acetonitrile at 80:20 from time 0 to 0.5 min. The ratio was linearly changed to 10:90 by 1 min and maintained until 4 min. The ratio was switched back to 80:20 by 4.5 min and maintained until the end of the run at 7 min. The mobile phase was degassed and filtered through a 0.22 μm Nylon 66 membrane prior to use and pumped at a flow rate of 220 μL/min. The MS/MS conditions were optimized by infusing each analyte (1 μg/mL) in the mobile phase, deionized water:acetonitrile 90:10) delivered at 220 μL/min. The MS was operated in single reaction monitoring mode (SRM) for quantification. The ESI was operated in the positive mode at a spray voltage of 4 kV and source CID of

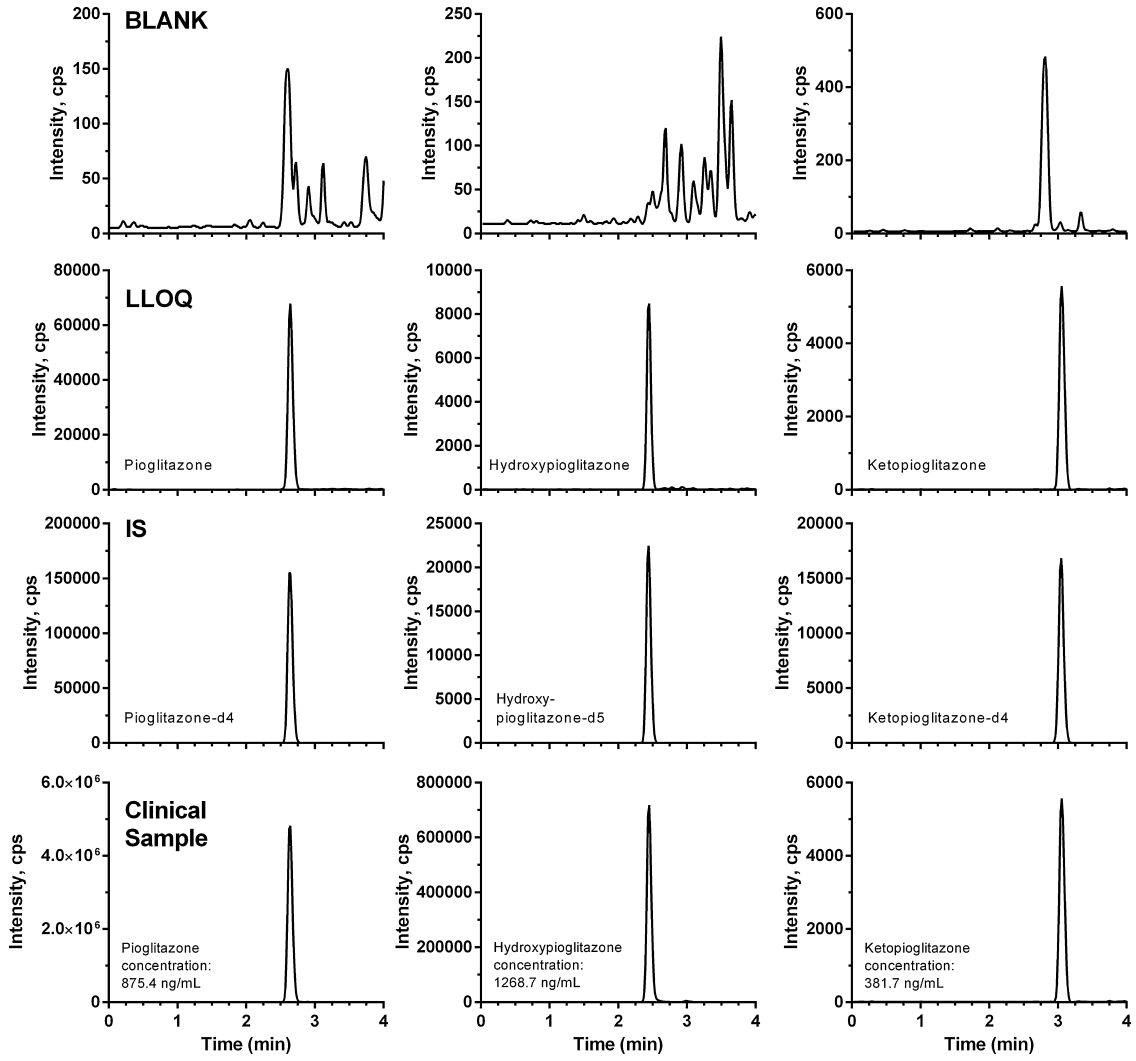

Fig. 2. Representative extracted ion chromatograms. Blank plasma, plasma spiked at the lower limit of quantitation (LLOQ; 10 ng/mL) or with the deuterated internal standard (IS), and plasma sample obtained from a study participant after chronic daily administration of pioglitazone 45 mg.

−3 V with a heated capillary temperature of 325 °C. The sheath and auxiliary gas was nitrogen, and the flow rates were set to 40 and 50 arbitrary units, respectively. The argon collision gas pressure was set to 1.5 mTorr and the collision energy was −33 eV. The MS/MS transitions monitored were *m/z* 357.1 → 134.0 for pioglitazone, *m/z* 373.1 → 150.0 for hydroxypioglitazone, *m/z* 371.0 → 148.0 for ketopioglitazone, *m/z* 361.1 → 138.0 for pioglitazone-d4, *m/z* 378.1 → 154.0 for hydroxypioglitazone-d5, and *m/z* 375.1 → 152.0 for ketopioglitazone-d4. The scan width was 1.0. The instrument was operated with a peak width (FWHM) set to *m/z* 0.70 at both Q1 and Q3. The scan time was 100 ms for each transition. Xcalibur® software version 1.4, service release 1 (Thermo Scientific, San Jose, CA, USA) acquired and processed the SRM data.

2.3. Standard preparation

Stock solutions of pioglitazone, hydroxypioglitazone, and ketopioglitazone were prepared in methanol at concentrations of 1, 10, and 100 μg/mL and used to prepare calibration standards and quality control (QC) samples. Stock solutions of each deuterated internal standard were prepared in methanol at a concentration of 250 ng/mL; the stock solutions of the three internal standards were mixed together. All stock solutions were stored at −20 °C. Calibration standards were prepared by spiking blank human plasma with varying quantities of the pioglitazone, hydroxypioglitazone, and ketopioglitazone stock solutions for the final concentrations of each analyte to be 10, 50, 100, 500, 1000, 1500, and 1800 ng/mL. The lower limit of quantitation (LLOQ) was considered to be the lowest calibration standard concentration. QC samples were similarly prepared at concentrations of 10 (LLOQ), 30, 800, and 1300 ng/mL. The standards and QCs were stored at −20 °C.

2.4. Sample preparation

Acetonitrile containing 1% formic acid (300 μL) and the combined internal standard solution (60 μL) were added to HybridSPE® Phospholipid 96-well plates (SUPELCO, Bellefonte, PA, USA), and then 100 μL plasma was pipetted into each well. The plate was mixed for 2 min at room temperature. After mixing, the plate was fitted with a vacuum collar and a 2 mL collection plate and filtered by vacuum 4 min. The resulting filtrate was diluted with 1500 μL deionized water and 10 μL was injected into the HPLC system.

2.5. Calibration and linearity

Calibration standards over the concentration range of 10–1800 ng/mL were analyzed in duplicate for three runs. The standards were deemed acceptable if both the precision, expressed

Table 1
Intra- and inter-run precision (relative standard deviation [RSD], %) and accuracy (relative error [RE], %) for quality control (QC) samples in human plasma. Eight replicates of each QC were analyzed in three separate validation batches ($n = 24$ total).

Analyte	Nominal concentration (ng/mL)	Intra-run	Inter-run	Accuracy (RE, %)
		Precision (RSD, %)	Precision (RSD, %)	
Pioglitazone	10 (LLOQ)	8.8	8.9	2.4
	30 (low QC)	6.2	6.4	2.1
	800 (medium QC)	5.3	5.3	−1.7
	1300 (high QC)	5.3	5.8	1.5
Hydroxypioglitazone	10 (LLOQ)	12.0	14.2	0.0
	30 (low QC)	7.9	7.9	5.8
	800 (medium QC)	6.8	7.2	−0.2
	1300 (high QC)	6.9	7.0	2.4
Ketopioglitazone	10 (LLOQ)	9.8	9.8	9.3
	30 (low QC)	7.6	8.3	1.3
	800 (medium QC)	5.3	5.6	−1.0
	1300 (high QC)	6.8	8.1	1.9

as percent relative standard deviation (RSD, %), and the accuracy, expressed as the percent relative error (RE, %), were within ±15%. The LLOQ was acceptable if the RSD and RE were within ±20%.

2.6. Precision and accuracy

QC samples at concentrations of 10 (LLOQ), 30 (low), 80 (medium), and 1300 (high) ng/mL were analyzed to determine the intra- and inter-run precision (RSD, %) and accuracy (RE, %) of the assay. Replicates ($n = 8$) at each QC concentration were analyzed in three batches run on separate days for the analysis of intra-run and inter-run precision and accuracy ($n = 24$ samples at each concentration). Means, standard deviations, RSD%, and RE% were calculated from the QC values by ANOVA [17].

2.7. Selectivity and stability

Selectivity was evaluated by processing and analyzing blank plasma samples obtained from six different human plasma lots. Carry-over was evaluated by injecting methanol after high calibration standards three times in a run. The autosampler stability of processed samples (post-preparative stability) was evaluated by analyzing QC samples immediately and 72 h after processing. After completing the initial analysis, the QC samples were stored in the autosampler at 10 °C for at least 72 h and then re-analyzed. The measured concentrations from both analyses were compared against freshly prepared samples. The stability after freeze-thaw cycles was determined by subjecting QC samples to three freeze-thaw cycles prior to processing, and comparing the results to the respective nominal concentrations.

2.8. Matrix effect and extraction efficiency

The potential for a matrix effect was evaluated by comparing the response values obtained from blank plasma samples spiked to QC concentrations after processing to those of deionized water spiked with an equivalent amount of analytes. Extraction efficiency was assessed by comparing response values of processed plasma QC samples to that of blank plasma samples spiked after processing with analytes to an equivalent concentration. The comparisons were made at low, medium, and high QC concentrations for analysis of both matrix effect and extraction efficiency ($n = 6$ at each concentration).

2.9. Application to plasma samples from a clinical trial

This method was used to measure plasma concentrations of pioglitazone and the two metabolites in samples obtained from patients who participated in a clinical study. These patients were diagnosed with nonalcoholic steatohepatitis and either type 2 diabetes mellitus or pre-diabetes (impaired fasting glucose or impaired glucose tolerance). Pioglitazone 45 mg/day was administered chronically for 18 months and samples were collected at the end of treatment. The clinical study objective was to evaluate the efficacy of pioglitazone as a treatment of nonalcoholic steatohepatitis. Each subject granted written informed consent, and the study was approved by the Institutional Review Board at the clinical study site. The blood samples were collected prior to the morning dose and processed and the resulting plasma was stored at −80 °C until analyzed.

3. Results and discussion

3.1. Chromatography

Representative extracted ion chromatograms of pioglitazone, hydroxypioglitazone, and ketopioglitazone obtained from the analysis of blank plasma, plasma spiked at the LLOQ or with the deuterated internal standard, and a plasma sample obtained from a study participant after chronic administration of pioglitazone 45 mg daily are shown in Fig. 2. Symmetrical peak shapes were obtained for all three analytes. The peak retention times were approximately 2.8 min for pioglitazone and pioglitazone-d4, 2.6 min for hydroxypioglitazone and hydroxypioglitazone-d5, and 3.2 min for ketopioglitazone and ketopioglitazone-d4.

3.2. Calibration and linearity

The calibration curve was constructed by linear regression with $(1/y^2)$ weighting and was linear for each analyte over a concentration range of 10–1800 ng/mL. Area ratios of analytes to the internal standards were used as the response measure for the calibration curve. Three batches of duplicate calibration curves were analyzed. The correlation coefficients (r^2) were greater than 0.99 for all three analytes and for all three validation batches.

3.3. Precision and accuracy

The intra- and inter-run precision (RSD, %) and accuracy (RE, %) calculated for pioglitazone, hydroxypioglitazone, and ketopioglitazone are shown in Table 1. The back-calculated concentrations based on the calibration curves were used at the LLOQ and the low, medium, and high QCs. The calculated precision and accuracy met the acceptance criteria for all three analytes.

3.4. Selectivity and stability

When six different plasma lots were analyzed for pioglitazone, hydroxypioglitazone, ketopioglitazone and the internal standards, no interfering peaks were observed. Any detected peaks from the sample injected following the highest calibration standard were less than 20% of a LLOQ peak size, and no carry-over effect was evident for the three analytes. Autosampler stability was determined by comparing results for samples that were analyzed immediately and at least 72 h after processing. The back-calculated concentrations of the samples stored in the autosampler at a temperature of 10 °C did not deviate by more than 15% at each QC level. Thus, the processed samples remained stable for at least 72 h at the autosampler temperature. The back-calculated QC concentrations were within 15% of previously measured concentrations after three freeze-thaw cycles, and no degradation of pioglitazone, hydroxypioglitazone, and ketopioglitazone was detected.

3.5. Matrix effect and extraction efficiency

Peak areas of six plasma samples spiked with pioglitazone, hydroxypioglitazone, and ketopioglitazone were compared to those of six deionized water samples spiked with the three analytes to evaluate for matrix effect at each QC level. There was less than 5% difference in the response at each QC level for all three analytes, and no evidence of matrix effect was found. Extraction efficiency was analyzed by spiking plasma samples before and after the protein precipitation and filtering. The measured recovery was 89.6 ± 8.2% for pioglitazone, 104.6 ± 11.9% for hydroxypioglitazone, and 87.8 ± 5.9% for ketopioglitazone (mean ± SD). Neither matrix effect nor extraction efficiency was shown to bias any quantitative measurements of the three analytes.

3.6. Application to plasma samples from a clinical trial

The method described was successfully used to support a clinical study in which pioglitazone was chronically administered to patients with nonalcoholic steatohepatitis. The chromatograms of pioglitazone and its active metabolites detected from a representative patient sample are shown in Fig. 2. This method was suitable for the analysis of human plasma samples obtained during the clinical trial. The average steady-state concentrations of pioglitazone, hydroxypioglitazone, and ketopioglitazone in 10 patients taking pioglitazone 45 mg/day were: 381.4 ng/mL (range 66.8–1624.0 ng/mL), 303.7 ng/mL (range 488.5–1380.0 ng/mL), and 119.2 ng/mL (range 85.4–640.5 ng/mL), respectively. The patient blood samples were collected prior to the morning dose. The patients were on a stable-dose and assumed to be at steady state. However, the exact time relative to the last dose was not verified or standardized for these clinical samples. This likely contributes to the large variability observed in the measured concentrations and is the subject of current studies.

4. Conclusion

We have validated a sensitive and selective LC–MS/MS method for quantitative and simultaneous determination of pioglitazone and the two major active metabolites, hydroxypioglitazone and ketopioglitazone, in human plasma. This method utilizes straightforward protein precipitation and filtration in a 96-well format; it takes less than an hour sample processing. This method is currently being used to measure concentrations of pioglitazone and its active metabolites in samples from a clinical trial.

References

[1] B. Cariou, B. Charbonnel, B. Staels, Trends Endocrinol. Metab. 23 (2012) 205.
[2] H. Yau, K. Rivera, R. Lomonaco, K. Cusi, Curr. Diab. Rep. 13 (2013) 329.
[3] B. Radhika, S. Vijayakumar, Int. J. Pharm. Sci. Lett. 2 (2012) 1.
[4] D.A. Eckland, M. Danhof, Exp. Clin. Endocrinol. Diab. 108 (2000) S234.
[5] M. Hanefeld, Int. J. Clin. Pract. (2001) 19.
[6] T. Jaakkola, J. Laitila, P.J. Neuvonen, J.T. Backman, Basic Clin. Pharmacol. Toxicol. 99 (2006) 44.
[7] X.S. Lai, L.P. Yang, X.T. Li, J.P. Liu, Z.W. Zhou, S.F. Zhou, Curr. Drug Metab. 10 (2009) 1009.
[8] P. Sripalakit, P. Neamhom, A. Saraphanchotiwitthaya, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 843 (2006) 164.
[9] K. Yamashita, H. Murakami, T. Okuda, M. Motohashi, J. Chromatogr. B: Biomed. Appl. 677 (1996) 141.
[10] W.Z. Zhong, M.G. Williams, J. Pharm. Biomed. Anal. 14 (1996) 465.
[11] M.B. Shankar, V.D. Modi, D.A. Shah, K.K. Bhatt, R.S. Mehta, M. Geetha, B.J. Patel, J. AOAC Int. 88 (2005) 1167.
[12] Z.J. Lin, W. Ji, D. Desai-Krieger, L. Shum, J. Pharm. Biomed. Anal. 33 (2003) 101.
[13] Y.J. Xue, K.C. Turner, J.B. Meeker, J. Pursley, M. Arnold, S. Unger, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 795 (2003) 215.
[14] B. Jagadeesh, D.V. Bharathi, C. Pankaj, V.S. Narayana, V. Venkateswarulu, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 930 (2013) 136.
[15] M. Wang, I.R. Miksa, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 856 (2007) 318.
[16] M. Uchida, M. Kanazawa, A. Ogiwara, H. Sezaki, A. Ando, Y. Miyamoto, Int. J. Mol. Sci. 14 (2013) 19716.
[17] B. DeSilva, W. Smith, R. Weiner, M. Kelley, J. Smolec, B. Lee, M. Khan, R. Tacey, H. Hill, A. Celniker, Pharm. Res. 20 (2003) 1885.