Jordan Journal of Pharmaceutical Sciences, Volume 7, No. 3, 2014

Simple and Rapid Quantification of Pioglitazone and Hydroxy Pioglitazone in Human Plasma Using Liquid Chromatography Coupled with Tandam Mass Spectrometry

***R. Revathi*[*1], *Bipinchandra Shinde*[1], *Shankar Pawar*[2]**

[1] Department of Pharmaceutical Analysis, The Erode College Pharmacy and Research Institute, Tamil Nadu, India.
[2] Lupin Bioresearch Center, Pune.

ABSTRACT

A liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method was developed for the simultaneous determination of Pioglitazone (PIO) and Hydroxy Pioglitazone (PIO M IV) in human plasma. Analytes were separated from plasma by protein precipitation extraction technique with 0.1% Formic Acid in acetonitrile as precipitant. Chromatographic separation was performed on a Thermo Hypurity 50 x 4.6 mm, 5µ-C18 column with the mobile phase consisted of 0.10% Formic acid in acetonitrile: 10mm Ammonium acetate (70:30). A tandem mass spectrometer equipped with atmospheric pressure chemical ionization source was used as detector and operated in the positive ion mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions m/z 357.2, 134.1 and m/z 373.1, 149.9 for PIO and PIO M IV respectively. The method showed a good linearity in a concentration range of 20.48 - 40000 ng/mL for PIO, and 10.29 - 2000 ng/mL for PIO M IV. The intra and inter-day precision was less than 15% and the absolute recovery was above 95%. This method was selective and rapid, sensitive for investigating drug concentrations in clinics.

Keywords: Pioglitazone, Protein precipitation, Hydroxy-derivative (M-IV), LC-MS/MS.

1. INTRODUCTION

Pioglitazone (PIO) is an oral anti diabetic agent that has been shown to affect abnormal glucose and lipid metabolism associated with insulin resistance by enhancing insulin action on peripheral tissues in animal models. PIO is commonly used for the treatment of diabetes mellitus type II. It exerts its anitidiabetic activity through selective stimulation of nuclear receptor peroxisone proliferator-activated receptor gamma (PPAR-gamma). Chemically, PIO is, 5-{4-[2-(5-ethyl-2-pyridyl) ethoxy] benzyl}-2, 4-thiazolidinedione hydrochloride salt.

PIO is extensively metabolized by hydroxylation and oxidation. At steady-state, Hydroxy Pioglitazone (PIO M-IV) reaches serum concentrations equal to or greater than PIO. Analysis of PIO and its metabolites, in formulation and biological fluids, by high performance liquid chromatography (HPLC) with ultraviolet detection, LC-MS/MS and HPTLC, have been reported in the literature[1-8]. Nonetheless, the previously published methods lack both specificity and sensitivity. In addition, the analysis time is considerably high with enormous extraction procedure to separate the active drug from the biological fluid. Thus, the aim of the present study is to develop a simple, economic, precise, and sensitive HPLC

* revathethiraj@gmail.com
Received on 23/3/2014 and Accepted for Publication on 10/8/2014.

method, based on MS-MS detection, with shorter run time for the quantitation of PIO and PIO M IV in human plasma.

Experimental:

Reagents and Instumentation

PIO, PIO (D4), PIO M IV (D4) were procured from Vivan life sciences. PIO M IV was procured from Clearsynth lab. The standards procured have more than 99% purity. The chemical structures of the above compounds are shown in Figure 1 (a-d). Acetonitrile (HPLC grade), ammonia, ammonium acetate, methanol (HPLC grade) were purchased from Merck (Country, City) while Water (HPLC grade) was obtained from a Milli-Q water purification system (Millipore, Milford, USA). All reagents were of analytical grade.

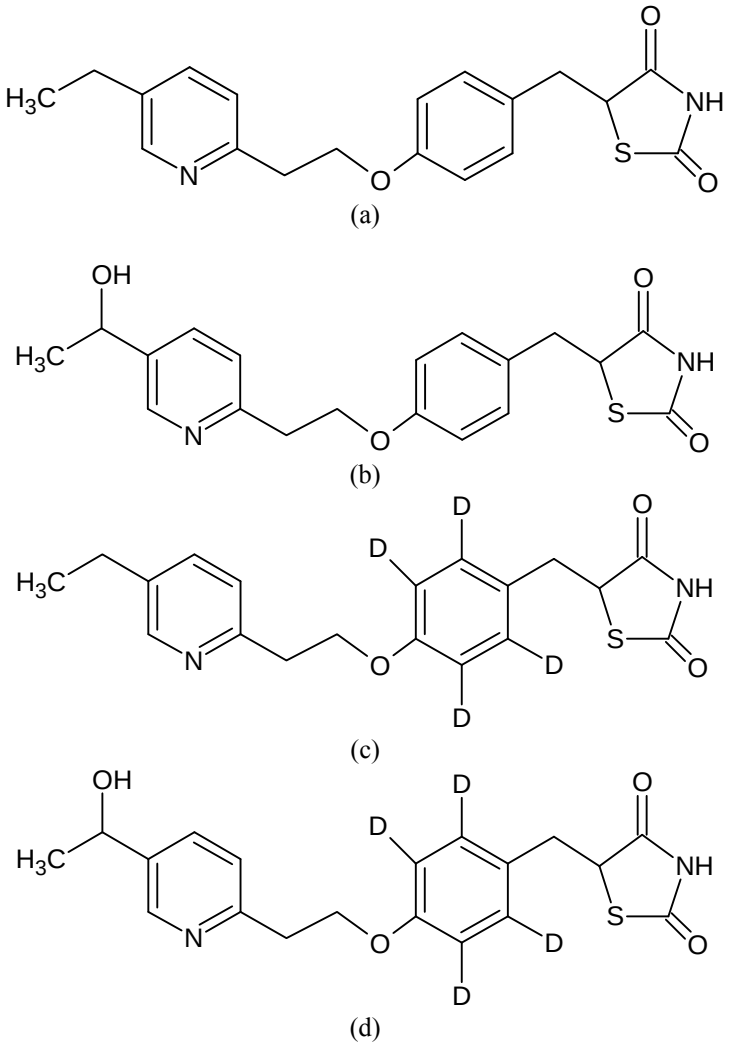


Figure 1. Chemical Structure of a) Pioglitazone, b) Hydroxy Pioglitazone, c) Pioglitazone D4, d) Hydroxy Pioglitazone D4

Analysis was performed on a QPXE Waters with APCI ionisation source using multiple reaction monitoring (MRM) detection. The isocratic HPLC mobile phase composed of 10 % formic acid, as mobile phase A, and 10 mm ammonium acetate buffer, as mobile phase B, in the ratio of 70:30. The mobile phase was

freshly prepared for each run, filtered through 0.45 µm filters, and degassed in ultrasonicator for 30 min before use. Flow rate was set at 0.5 mL/min and column oven temperature was maintained at 40 ± 2°C. The injection volume was 5.0 µL and the total run time was 4.50 min.

The mass spectrometer was operated in MS-MS mode using MRM of the transitions m/z 357.2 > 134.1 for PIO and 373.1 > 149.9 for PIO M IV.

Preparation of calibration curve (CC) standards and quality control (QC) samples

Working calibration standards were spiked freshly in plasma to produce concentration series of 20.58, 41.16, 196.0, 392.0, 784.0, 1568.0, 2240.0, 3200.0, 4000.0 ng/mL. The CC samples were analyzed along with the QC samples for each batch of plasma samples. The quality control samples were spiked in the range of 2.888 to 150.0 µg/mL. All the prepared plasma samples were stored at -70°C.

Sample extraction by protein precipitation method

About 50.0µL of internal standard (IS) dilution (about 30 + 20 µg/mL of Pioglitazone D4 (PD4) + Hydroxy Pioglitazone D4. (PM-IV D4)) was added into the pre-labelled tubes except the standard blank and pre-dose (without IS) samples. Accurately 200.0µL of plasma blank, CC standards and QC samples were aliquoted into pre-labelled tubes and vortexed to mix. Accurately, 1.0mL of 0.10% formic acid in acetonitrile was added to each sample tube and mixed before centrifugation at 12000 rpm for 10 minutes at 10 ± 3°C. The supernatant was transferred into a pre-labelled auto sampler vial.

Method validation

The validation of the above method was carried out as per US FDA guidelines. The parameters determined were selectivity, matrix effect, linearity, precision, accuracy, recovery, stability, and dilution integrity. Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma obtained from six different sources including one lipemic and one hemolyzed plasma. Sensitivity was determined by analyzing six replicates of plasma samples spiked with the lowest level of the calibration curve concentrations. Matrix effect was verified with six different lots of K2-EDTA plasma. Three replicate samples, each of LQC and HQC, were prepared from different lots of plasma (36 QC samples in total).

For linearity evaluation, standard calibration curves containing at least nine points (non-zero standards) were plotted in range of 20.31–4014.39 ng/mL for PIO. In addition, blank plasma samples were also analyzed to confirm the absence of direct interferences. *Inter*-day precision and accuracy were determined by the analysis of six replicates at four different QC levels on three different days. *Intra*-day precision and accuracy were determined by the analysis of six replicates at four different QC levels of five different runs in the same day. Recoveries of PIO and PIO M IV were determined by comparing extracted and unextracted samples respectively. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. Six replicates, each at a concentration of about 1.5 times of the uppermost calibration standard, were diluted two and four folds with blank plasma. The diluted samples were processed and analyzed.

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8°C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (09 hr 55 min), processed samples stability (Autosampler stability for 48 hr 05 min, reinjection stability for 46hrs), freeze thaw stability (four cycles) were performed at LQC and HQC levels using six replicates at each level.

Results and discussion

Method development

Mass parameters were tuned for both analytes. Good response was found in the positive ionization mode. Data in the MRM mode were considered, which showed better selectivity. Chromatographic conditions, especially the

composition of the mobile phase and the specifications of different columns, were optimized through several trials to attain good resolution, increase the intensity of analytes′ signals, and achieve short run times. The presence of a small amount of formic acid in the mobile phase enhanced the detection of analytes. It was found that a mixture of 0.1% formic acid in acetonitrile and 10 mM Ammonium acetate (70:30, v/v) could achieve this purpose and was finally adopted as the mobile phase. Thermo Hypurity (50 x 4.6mm), 5µ column gave good peak shapes and response even at lowest concentration levels for both the analytes and IS. The mobile phase was operated at a flow rate of 0.5mL/min. The retention time of PIO and PIO M IV was low enough (1.4 and 1.2 min, respectively) to allow a shorter run time of 4.50min. A simple protein precipitation extraction technique was employed for the sample preparation that provided high recoveries of the compounds.

Sensitivity

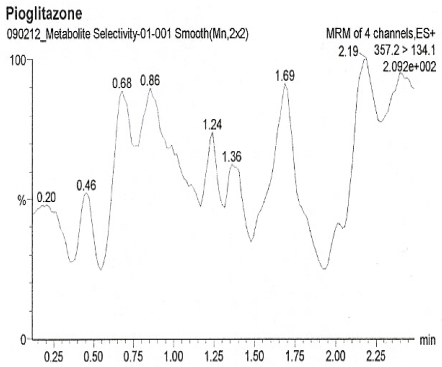
The lowest limit of reliable quantification for the analytes was set at the concentration of the lower limit of quantitation (LLOQ). The precision and accuracy at LLOQ concentration were found to be 6.99% and 90.67% for PIO, 11.63% and 95.92% for PIO M IV.

Matrix effect

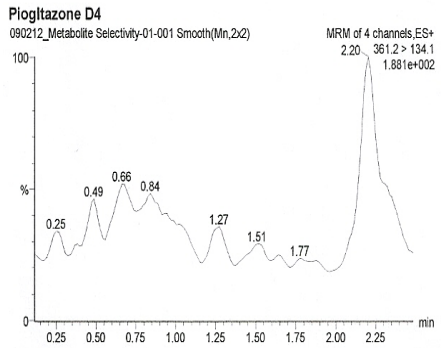
No significant matrix effect was observed in all the six batches of human plasma for the analytes at low and high quality control concentrations. The % coefficient of variation (CV) of precision and % nominal of accuracy for PIO at LQC concentration were found to be 2.84% and 95.55%, and at HQC level they were 2.49% and 95.58%, respectively. Similarly, the precision and accuracy for PIO M IV at LQC concentration were found to be 4.93% and 98.28%, and at HQC level they were 4.11% and 97.85%, respectively.

Selectivity and chromatography

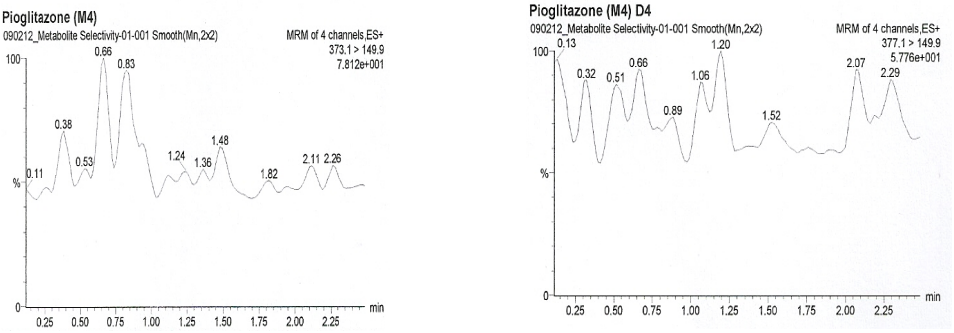
The degree of interference by endogenous plasma constituents with the analytes and the IS was assessed by inspection of chromatograms derived from processed blank plasma sample. As shown in Figure 2 (A and B), no significant direct interference in the blank plasma was observed, however, traces were observed from endogenous substances in drug free plasma at the retention time of the analytes.



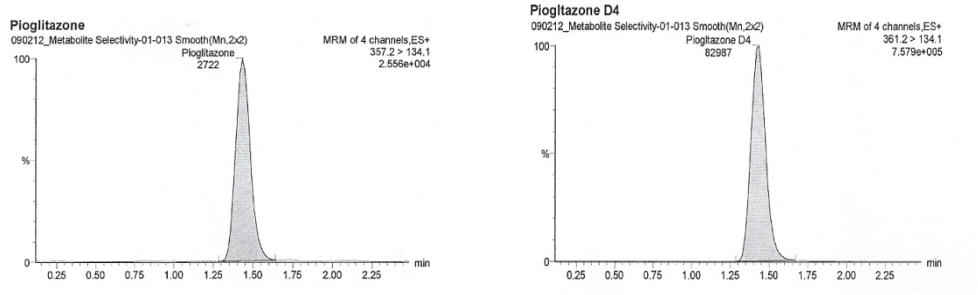
A) Chromatogram for blank



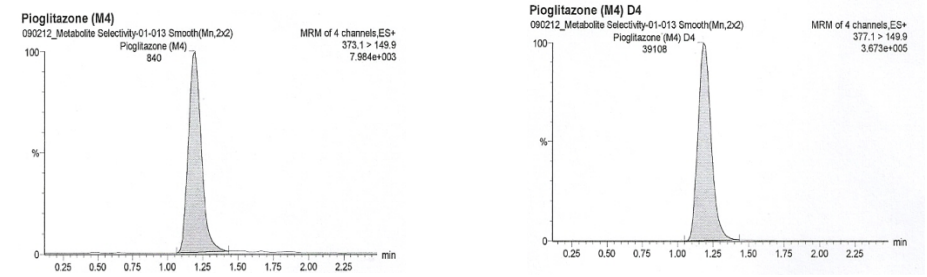
Chromatogram for blank + IS



B) Chromatogram for blank Chromatogram for blank + IS



C) LLOQ for Pioglitazone LLOQ for Pioglitazone D4 [IS]



LLOQ for Hydroxy Pioglitazone LLOQ for Hydroxy Pioglitazone D4 [IS]

Figure 2. Typical MRM chromatograms of Pioglitazone (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C)

Linearity

The nine point calibration curve was found to be linear over the concentration range of 20.31– 4014.39 ng/mL for PIO and 10.290–2000.0 ng/mL for PIO M IV. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration detector response relationship for both analytes in human plasma. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.99.

***Precision and accuracy*:**

As shown in Table 1 and 2, the %CV of precision and % nominal of accuracy of each analyte in the intra and inter day runs were within ±15% at LQC, MQC and HQC concentrations and within ±20% at LLOQ quality control sample (QCs).

Table 1: *Intra*-assay and *Inter*-assay precision and accuracy result for PIO

Pioglitazone Conc (ng/mL)	Precision (%CV)		Accuracy (%)	
	Intra-assay*	Inter-assay**	Intra-assay*	Inter-assay**
20.325 (LLOQ QC)	2.63	6.99	89.92	90.67
56.457 (LQC)	1.43	2.84	98.33	95.55
1660.510 (MQC)	1.26	2.77	99.90	95.93
3168.913 (HQC)	1.23	2.49	99.22	95.58

(*n=5, **n=3)

Table 2: *Intra*-assay and *Inter*-assay precision and accuracy result for PIO M IV

Hydroxy Pioglitazone Conc (ng/mL)	Precision (%CV)		Accuracy (%)	
	Intra-assay*	Inter-assay**	Intra-assay*	Inter-assay**
10.326 (LLOQ QC)	4.87	11.63	102.63	95.92
28.682 (LQC)	4.37	4.93	101.58	98.28
843.588 (MQC)	1.85	4.15	100.96	99.03
1609.901 (HQC)	2.91	4.11	99.80	97.85

(*n=5, **n=3)

***Acceptance Limits*[9]**

%CV & % nominal should be <15% and <115% for LQC, MQC & HQC, for LLOQ QC the %CV and % nominal should be <20% and <120%, respectively.

Extraction efficiency

Six replicates at low, medium and high quality control concentrations for PIO and PIO M IV were prepared for recovery determination. The recoveries of analytes and IS were good and reproducible. The mean overall recoveries (with the precision range) of PIO, PIO M IV and IS (PIO D4 & PIO MIV D4) were 98.95%, 98.50 % and 98.94% & 98.2 %, respectively.

Dilution integrity

The upper concentration limits can be extended to 6519ng/mL for PIO and 3440ng/mL for PIO M IV with screened human blank plasma. The mean back calculated concentrations for ½ and ¼ dilution samples were within 85–115% of their nominal value. The coefficients of variation (%CV) for ½ and ¼ dilution samples were less than 10%.

Stability studies

Different stability experiments were carried out at bench top stability (10h), autosampler stability (48h 5 min), repeated freeze thaw cycles (four cycles) and reinjection stability (46h 50 min), the mean % nominal

values of the analytes were within the limit and given in Table 3.

Table 3: Stability studies data for PIO and PIO M IV

Conditions	% Stability		
	Level	PIO	PIO M IV
Short term stock solution stability (11 hrs 21 min)	HQC	95.89	97.77
	LQC	100.24	101.51
Short term spiking solution stability (06 hrs 03 min)	HQC	101.84	97.96
	LQC	99.50	100.79
Bench top stability (09 hrs 55 min)	HQC	97.89	100.16
	MQC	98.68	99.41
	LQC	98.52	101.03
Auto sampler stability (48h 05 min)	HQC	99.61	101.71
	MQC	102.17	102.52
	LQC	102.41	101.21
Freeze thaw stability (-30°C)	HQC	101.68	99.07
	MQC	98.81	97.22
	LQC	103.32	103.94
Freeze thaw stability (-75°C)	HQC	101.05	96.10
	MQC	101.8	99.96
	LQC	102.73	105.62

CONCLUSIONS

The LC-MS/MS assay method described in this paper is rapid, simple, specific and sensitive for quantification of PIO and PIO M IV in human plasma and is fully validated as per the FDA guidelines. To the best of our knowledge, this is the first report on simultaneous assay of PIO and PIO M IV in any of the matrix without compromising on the reported sensitivity for each analyte. The method was found to be suitable for pharmacokinetic studies in humans. The simple protein precipitation method gave consistent and reproducible recoveries for the analytes from plasma. The method provided good linearity. A sample turn over rate of less than 4.5 min makes it an attractive procedure in high throughput bioanalysis of PIO and PIO M IV**.**

ACKNOWLEDGMENTS

The authors are highly thankful to Lupin Bioresearch Center, Pune, India for providing necessary facilities for carrying out this study.

REFERENCES

(1) Zhongping J. L., Weihua Ji, Daksha D. K., and Linyee S. Simultaneous determination of Pioglitazone and its two active metabolites in human plasma by LC/MS/MS. *J. Pharm. Biomed. Anal.* 2003; 33:101-108.

(2) Vijaya K. K., Nageswara Rao P., Jaswanth K. I., and J.V.L.N. Seshagiri Rao. Simultaneous determination of Pioglitazone and Candesartan in human plasma by LC-MS/MS and its application to a human pharmacokinetic study. *Journal of Pharmaceutical Analysis.* 2012; 2 :167-173.

(3) Kenji Y., Hiromi M., Teruaki O., and Michio M. High-performance liquid chromatographic determination of Pioglitazone and its metabolites in human serum and urine. *J. Chromatogr B: Biomed Sci Appli*. 1996; 677:141-146.

(4) Meeta A. J., Pandya S. S., and Vidyasagar G. A simple

and sensitive HPTLC method for estimation of Pioglitazone in bulk and tablet dosage forms. *Asian J. Research Chem.* 2009; 2: 207-209.

(5) Lakshmi K. S., Rajesh T., and Shrinivas S. Simultaneous determination of Metformin and Pioglitazone by reversed phase HPLC in pharmaceutical dosage forms. *International Journal of Pharmacy and Pharmaceutical Sciences.* 2009; 3: 2011-2017.

(6) Xuea Y. J., Kenneth C. T., Jeff B. M., Janice P., Mark A., and Steve U. Quantitative determination of Pioglitazone in human serum by direct-injection high-performance liquid chromatography mass spectrometry and its application to a bioequivalence study. *Journal of Chromatography B.* 2003; 795: 215–226.

(7) Hofmann C. A., Edwards C. W., and Colca J. R. Treatment of insulin-resistant mice with the oral antidiabetic agent pioglitazone: evaluation of liver GLUT2 and phosphoenolpyruvate carboxykinase expression. *Endocrinology.* 1992; 130: 735–740.

(8) Pawan K. B., Prabhat K. S., and Deepti J. Spectrophotometric estimation of Pioglitazone hydrochloride in tablet dosage form. *Asian Journal of Pharmaceutics.* 2008; 2: 225-227.

(9) Guidance for industry: Bioanalytical Method Validation, Centre for Drug Evaluation and Research (CDER) in cooperation with Centre for Veterinary Medicine (CVM) and Food and Drug Administration (FDA).

استحداث طريقة للقياس السريع والبسيط للبيوجليتازون وهيدروكسي بيوجليتازون في البلازما باستخدام الكروماتوغرافي الطيفي الشامل السائل

ريفاثي [1]، شيندي [1]، باوار [2]

[1] كلية إيرود للصيدلة، الهند.

[2] مركز لوبين للأبحاث البيولوجية، الهند.

ملخص

لقد تم تطوير طريقة باستخدام الكروماتوغرافي الطيفي الشامل السائل، (/ MSLC–MS) لتحديد تركيز دوائي البيوجليتازون وهيدروكسي بيوجليتازون في البلازما البشرية في نفس الوقت ولنفس العينة. تم فصل البلازما من التحاليل بوساطة تقنية استخراج البروتين عن طريق ترسيبه بوساطة حمض الفورميك بنسبة 0.1٪ في الأسيتونيتريل. تم تنفيذ الفصل الكروماتوغرافي باستخدام الحرارة كالآتي: Hypurity 50 × 4.6 مم، بحيث استخدم عمود C18–µ5 مع السائل المتحرك المكون من حمض الفورميك 0.10٪ مذاب في الأسيتونيتريل و10 ملي خلات الأمونيوم (70:30). تم استخدام مطياف الكتلة جنبا إلى جنب مع مصدر للضغط الجوي والتأين الكيميائي حيث استخدم الأخير للكشف عن وضع الأيون الإيجابي وتشغيله. تم إجراء القياس الكمي باستخدام مراقبة التفاعلات المتعددة (MRM) لقياس التحولات m/z 357.2, 134.1 .m/z 373.1, 149.9لدوائي البيوجليتازون وهيدروكسي بيوجليتازون على التوالي. أظهرت الطريقة الجديدة أن هناك أسلوباً خطياً جيداً لتركيز (- 20.48) 40000 ng/mL لبيوجليتازون، وتركيز (10.29 - 2000 ng/mL) لهيدروكسي بيوجليتازون. كانت الدقة داخل وبين الأيام أقل من 15٪ وكان الاسترجاع المطلق يزيد عن 95٪. كانت هذه الطريقة انتقائية وسريعة وحساسة لفحص تركيز الدواء في العيادات.

الكلمات الدالة: بيوجليتازون، مشتقة هيدروكسي (M-IV).

تاريخ استلام البحث 2014/3/23 وتاريخ قبوله للنشر 2014/8/10.