Short communication

A high performance liquid chromatography method for simultaneous determination of rosiglitazone and gemfibrozil in human plasma

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ABSTRACT

A high performance liquid chromatography (HPLC) method was developed for the simultaneous determination of rosiglitazone and gemfibrozil in human plasma using α-asarone as an internal standard (IS). Plasma samples were pretreated by protein precipitation. The analytes were separated on a Macherey–Nagel Nucleodur C_{18} (250 mm × 4.6 mm, 5 μm) column using acetonitrile and 30 mmol/l ammonium acetate solution (including 0.1% methanoic acid) as mobile phase which was delivered at 1.2 ml/min. A gradient elution program was adopted to adjust proportion of solvent in mobile phase. A time program was used to regulate conditions of fluorescence detection. The method was validated over the concentration range of 5.0–751.3 ng/ml for rosiglitazone and 0.5–75.4 μg/ml for gemfibrozil with acceptable accuracy, precision and extraction recoveries. This method is suitable for routine therapeutic drug monitoring and pharmacokinetic interaction study of the two drugs.

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1. Introduction

Rosiglitazone (RSG), an oral antidiabetic agent of the thiazolidinedione class, exerts its effect through activating peroxydase proliferation activation receptor-γ. It has received regulatory approval for the treatment of type 2 diabetes mellitus (T2DM) in both the monotherapy and the therapy in combination with other oral antidiabetic agents for its advantages of the therapeutic profile [1,2]. Gemfibrozil, a lipid-lowering drug, is widely used to lower high serum triglyceride concentrations in patients with T2DM. Studies have found that cytochrome P450 (CYP) 2C8 is primarily responsible for the metabolism of RSG in human liver, with minor contributions for CYP2C9 [3]. Gemfibrozil inhibits both CYP2C8 [4] and CYP2C9 [5]. Therefore, gemfibrozil may increase efficacy or risk of concentration-dependent adverse effects of RSG by inhibiting its metabolism when they are used together [6]. However, up to now, no analytical method has been reported on the simultaneous determination of the two drugs in human plasma. For studying the pharmacokinetic interaction of the two drugs, Niemi et al. utilized different methods to determine them respectively (HPLC method with fluorescence detection for RSG; HPLC method with ultraviolet detection for gemfibrozil) [6], It made the detection procedure tedious and time-consuming. To simplify the process, we decided to establish a method to determine them simultaneously. Some analytical methods have been presented for individual measurement of RSG [7–17] and gemfibrozil [18–21], such as high performance liquid chromatography with ultraviolet (HPLC-UV) detection, fluorescence detection (HPLC-FD) and liquid chromatography-tandem mass spectrometry (HPLC-MS -MS). But the drawbacks of the HPLC-UV methods involving the low sensitivity, long analysis time and complex sample preparation limit their usage in routine therapeutic drug monitoring (TDM) and pharmacokinetic interaction study of the two drugs. Assay by using a HPLC-MS-MS instrument could achieve higher sensitivity and faster detection, but the instrumentation is rather expensive. So we attempted to develop a HPLC-FD method to overcome the above limitations. This paper describes a simple, sensitive HPLC-FD method which is suitable for TDM and pharmacokinetic interaction study of the two drugs in patients.

2. Experimental

2.1. Chemicals and reagents

Rosiglitazone (99.8%) was purchased from Beijing Caomen chemical engineering limited company (Beijing, China). Gemfibrozil (>99.9 %) was purchased from National Institute for the Control of Pharmaceutical and Biological products (Beijing, China). α-asarone (99.8%) was purchased from Guangxi Liuzhou pharmaceutical factory (Guangxi, China). HPLC grade methanol and acetonitrile were purchased from Merck (Germany). Ammonium acetate and methanoic acid were analytical grade. Blank human plasma was obtained from the Blood Center of Shanghai (Shanghai, China) or from healthy volunteers.

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2.2. Instrumentation

The HPLC system was a Shimadzu LC-10A series (Kyoto, Japan), equipped with a LC-10AT*vp* pump, a RF-10A*xL* fluorescence detector, a SCL-10A*vp* controller, a SIL-10AD*vp* autosampler, and a CTO-10A*vp* incubator. Data collection and processing were performed with Class-VP6.2 chromatographic workstation (Shimadzu Corporation, Kyoto, Japan).

2.3. Chromatographic conditions

The analytes were separated on a Macherey–Nagel Nucleodur C_{18} (250 mm × 4.6 mm, 5 μm) column with column temperature 45 °C. The gradient mobile phase consisted of acetonitrile (A) and 30 mmol/l ammonium acetate solution containing 0.1% (v/v) methanoic acid (B). The starting mobile phase consisted of 52% A and 48% B, changed gradually to 64% A and 36% B in 11 min, then returned to the initial condition in 1 min and re-equilibrated for 4 min. The run-time was 16 min at a flow rate of 1.2 ml/min. The time program was performed as follows: fluorescence detector was set at 250 nm (excitation wavelength, λ_{ex}) and 370 nm (emission wavelength, λ_{em}) to detect RSG during 0.00–7.70 min, 265 nm (λ_{ex}) and 365 nm (λ_{em}) to detect α-asarone during 7.70–10.40 min and 242 nm (λ_{ex}) and 300 nm (λ_{em}) to detect gemfibrozil during 10.40–16.00 min. The values of gain and sensitivity were 3 and 3 during 0.00–7.70 min, 2 and 3 during 7.70–10.40 min, and 2 and 2 during 10.40–16.00 min.

2.4. Preparation of standard solutions, calibration standards and quality control samples

The primary stock solutions of rosiglitazone (199.0 μg/ml), gemfibrozil (3014.0 μg/ml) and α-asarone (IS, 102.0 μg/ml) were prepared by dissolving appropriate amounts of the pure substances in methanol. The IS stock solution was further diluted in methanol to yield a 200 ng/ml working solution. The stock solutions of RSG and gemfibrozil were diluted in methanol to produce appropriate working solutions before using. All the standard solutions were stored at 4 °C.

Blank plasma was spiked with working solutions of the drugs to achieve the following calibration standard concentrations: RSG: 5.0, 15.0, 30.0, 60.1, 150.3, 300.5, 751.3 ng/ml; gemfibrozil: 0.50, 1.5, 3.0, 6.0, 15.1, 30.1, 75.4 μg/ml. The concentration ranges were selected based on reported analysis of RSG [13] and gemfibrozil [18]. Plasma quality control samples (QCs) which were run in each assay were prepared in the same way, the concentrations of QCs were 15.0, 60.1, 300.5 ng/ml for RSG and 1.5, 6.0, 30.1 μg/ml for gemfibrozil. Calibration standards and QCs were stored at −20 °C.

2.5. Sample pretreatment

All calibration standards and QCs were pretreated as follows: The plasma was centrifuged at 3000 rpm for 5 min. 0.15 ml of supernatant, 15 μl of α-asarone working solution (IS, 200 ng/ml) and 0.45 ml of acetonitrile were put together and mixed vortically for 2 min, then the mixture was centrifuged at 9500 rpm for 10 min, 20 μl of the upper layer was drawn off carefully and injected for analysis.

2.6. Method validation

The method was validated by determination of linearity, the lower limit of quantitation (LLOQ), accuracy, precision, extraction recovery, selectivity and stability.

2.6.1. Linearity and LLOQ

Calibration was performed by a least squares linear regression of the peak-area ratios of the drugs to the IS versus the respective standard concentration (RSG: 5.0, 15.0, 30.0, 60.1, 150.3, 300.5, 751.3 ng/ml; gemfibrozil: 0.50, 1.5, 3.0, 6.0, 15.1, 30.1, 75.4 μg/ml). The lowest concentration on the standard curve with detector response five times greater than the blank human plasma was considered as the LLOQ. The analyte peak in LLOQ sample should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was determined as the concentrations with a signal-to-noise ratio of 3.

2.6.2. Accuracy and precision

Accuracy was estimated as the percentage of the measured concentration over the nominal concentration, accuracy was determined by repeated analysis of five spiked samples of RSG and gemfibrozil at each QC level. Relative standard deviation (R.S.D.) were calculated from QC values and used in the estimation of intra- and inter-day precision which were analyzed by five replicates of each QC level in one day and on three different days.

2.6.3. Extraction recovery

The extraction recovery was determined by comparing the peak areas of each compound after extraction with those obtained by direct injection of the same amount of analyte in standard solution. Replicate analysis ($n = 5$) of QCs at three concentration levels was used for determining extraction recovery of the assay.

### 2.6.4. Selectivity and stability

The selectivity of this method was investigated by preparing and analyzing six individual human blank plasma samples. Selectivity was assessed by comparing the chromatograms obtained from the sample spiked with standards of RSG and gemfibrozil with those obtained from blank human samples. Room temperature stability was analyzed at 0, 6, 12, 18, 24 h. Freeze-thaw stability was analyzed at room temperature after five repeated freeze-thaw cycles (−70 °C to room temperature), and permanent stability was analyzed using the QCs kept at −70 °C for 8 days.

3. Results and discussion

3.1. Chromatographic conditions and internal standard

If proportion of acetonitrile in mobile phase was fixed, the disparities in the retention times of RSG, IS and gemfibrozil would be too great, and moreover, retention time of gemfibrozil was so long that the total analysis time was more than 20 min. So we used a gradient elution program to shorten the retention time of gemfibrozil and total analysis time. With the gradient program, the retention times for RSG, IS and gemfibrozil were 5.75, 9.34 and 12.62 min respectively, and the total analyte time was 16 min. At the same time, this gradient elution program yielded sharp peaks to increase signal-to-noise ratio and sensitivity.

λ_{ex} and λ_{em} of RSG, IS and gemfibrozil were different, so we used the time program to adjust λ_{ex} and λ_{em} of every time period to detect the three compounds separately. In our study, the concentration ranges of RSG and gemfibrozil were 5.0–751.3 ng/ml and 0.50–75.4 μg/ml respectively. Difference of the highest quantitation concentrations of the two analytes was about 100 times and that of the lowest concentrations was just the same. The disparity between their concentrations would make ranges of their responses beyond the linear range of detector response and induce linear distortion, so we used the time program to resolve the problem. In the program, the sensitivity and gain of fluorescence detector in the corresponding time period were adjusted. The adjustment made ranges of their responses approximate and in the linear range of
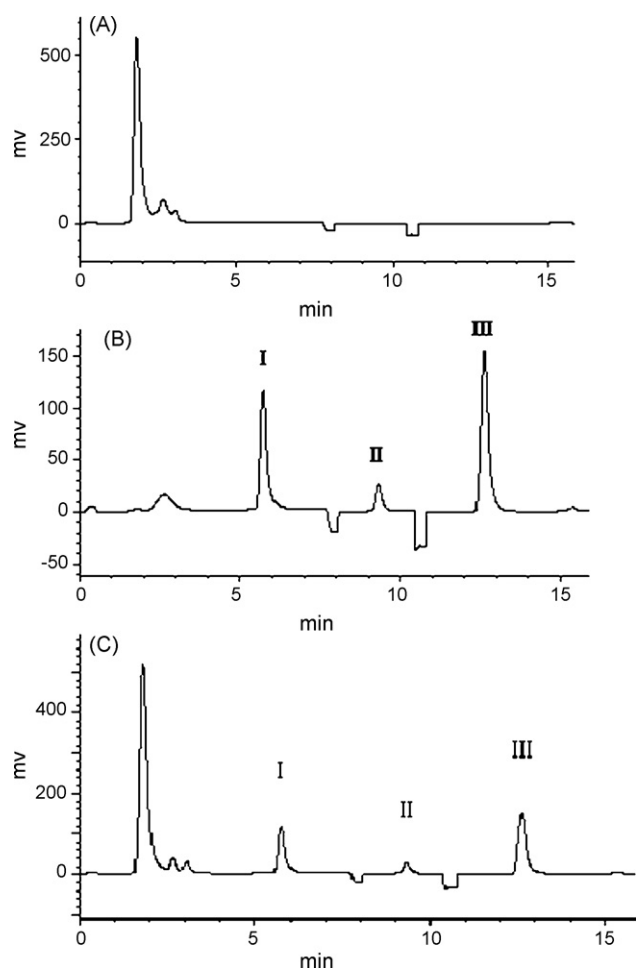
Fig. 1. Representative chromatograms of (A) blank human plasma sample, (B) standard solution containing 300.5 ng/ml RSG, 200 ng/ml IS and 30.1 μg/ml gemfibrozil, (C) blank human plasma spiked with 300.5 ng/ml RSG, 200 ng/ml IS and 30.1 μg/ml gemfibrozil. Peaks: (I) Rosiglitazone, (II) IS, and (III) Gemfibrozil.

detector response to avoid linear distortion. The response of the IS was also adjusted simultaneously with the program to avoid the mismatch of the response of the IS with that of RSG or gemfibrozil.

α-asarone was selected as the IS due to its fluorescence spectrum, suitable retention time and good chromatographic separation [22–24].

3.2. Optimized chromatography

The representative chromatograms of blank human plasma, standard solution and standard in blank human plasma are shown in Fig. 1. The peaks of interest were well separated and free from interference with endogenous substances. The retention times of RSG, α-asarone and gemfibrozil were 5.75, 9.34 and 12.62 min respectively. The foveation peaks at 7.70 and 10.40 min were caused by transformation of conditions of fluorescence detection and reset of the detector.

3.3. Linearity and LLOQ

Assay linearity was evaluated by calibration curves ranging from 5.0 to 751.3 ng/ml for RSG and 0.50–75.4 μg/ml for gemfibrozil. Using linear regression analysis, an excellent linear relationship between peak area ratio and concentrations was exhibited for RSG, $y = 71.951x + 1.188$, $r = 0.9998$ and for gemfibrozil, $y = 4.750x - 0.062$, $r = 0.9991$, where y refers to the peak area ratio of RSG or gemfibrozil

to the IS and x corresponds to the standard concentration of RSG or gemfibrozil, r is correlation coefficient. LLOQ were 5.0 ng/ml for RSG and 0.50 μg/ml for gemfibrozil, the lowest detectable limit of RSG and gemfibrozil were 2.0 ng/ml and 0.050 μg/ml respectively.

3.4. Accuracy and precision

Accuracy was within ±5% for RSG and ±10% for gemfibrozil. Accuracy of the three concentration levels (low, medium, and high: 15.0, 60.1, 300.5 ng/ml for RSG; 1.5, 6.0, 30.1 μg/ml for gemfibrozil) was 95.3%, 102.8% and 99.8% for RSG and 104.2%, 90.9% and 98.8% for gemfibrozil. The intra- and inter-day precision (R.S.D.) of the two analytes were less than 10.0%, the intra-day R.S.D. was 1.2%, 4.5% and 3.0% for RSG and 6.0%, 1.0% and 1.8% for gemfibrozil, the inter-day R.S.D. was 4.3%, 5.3% and 5.6% for RSG and 7.7%, 7.4% and 5.6% for gemfibrozil.

3.5. Extraction recovery

The extraction recoveries of the low, medium and high concentration were 85.6%, 82.4% and 79.2% for RSG and 87.9%, 85.1% and 80.2% for gemfibrozil separately.

3.6. Selectivity and stability

No endogenous interference with RSG, IS and gemfibrozil was observed in six different sources of blank plasma. The concentrations of RSG, gemfibrozil and IS did not change obviously in three different conditions (at room temperature for 24 h, −70 °C for 8 days, five repeated freeze-thaw cycles after), All the R.S.D. were less than 10%.

4. Conclusion

The main aim of the study was to establish a HPLC-FD method that was suitable for simultaneous determination of RSG and gemfibrozil in human plasma. The method presented here was proved to be simple, specific and accurate. To the best of our knowledge, this method meets the request of the present pharmacokinetic studies of the two drugs. Thus, the method suits for routine TDM and pharmacokinetic interaction study of RSG and gemfibrozil.

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