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Reversed-phase HPLC methods for purity test and assay of pioglitazone hydrochloride in tablets

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A reversed-phase gradient HPLC method was developed for the evaluation of pioglitazone hydrochloride (PG-HCl) in tablets. Limit of detection for PG-HCl was found to be 42 ng/ml. Analyses were performed on a C18 column (Symmetry C18, 5 μm, 250 × 4.6 mm), mobile phase was a mixture of ammonium formate buffer adjusted with formic acid to pH 4.1 and acetonitrile. Shortened purity method was used as the assay method. Methods were validated.

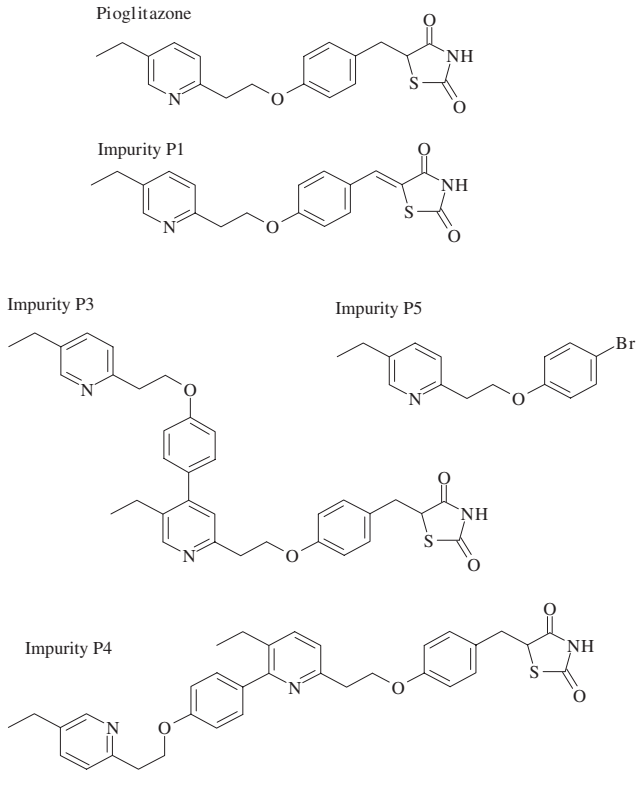
1. Introduction

Pioglitazone (PG) – (*RS*)-5-{4-[2-(5-Ethyl-2-pyridyl)-ethoxy]-benzyl}-2,4-thiazolidindione – is known as a substance with hypoglycemic effect and it is used for therapy of non-insulin-dependent diabetes mellitus (Castle et al. 1993; Hofmann et al. 1991; Hofmann et al. 1992; Ikeda et al. 1990; Kletzien et al. 1992; Kobayashi et al. 1992; Sohda et al. 1992). Several papers dealing with the determination of pioglitazone and its metabolites in human/dog serum and urine have been published (Yamashita et al. 1996; Zhong, Lakings 1989; Zhong, Williams 1996). There were found only two works dealing with the evaluation of related substances and assay of pioglitazone hydrochloride (PG-HCl) in pharmaceutical formulations (Chen et al. 2000; Radhakrishna et al. 2002).
Information needed for method validation was gathered from the guidelines (ICH guideline Q2A, ICH guideline Q2B) and also paper (Romanyshyn and Tiller 2001) provided useful information for method development. Due to a different approach in the evaluation of PG-HCl in tablets in this article than in already published works we propose these methods for the evaluation of PG-HCl.

2. Investigations, results and discussion

2.1. Purity method

Chromatographic conditions were optimized to make the method usable not only as HPLC-UV method, but after small changes as HPLC-MS method.
The initial mobile phase tested was a mixture of water and acetonitrile (50 : 50, v/v). The peaks of PG and impurity P1 were distorted and also the efficiency of separation of all compounds expressed by numbers of theoretical plates was poor. At the same time the retention times of impurities P3–5 were heigh. Therefore a gradient elution was subsequently used.
The second step was testing of a buffered mobile phase. To ensure the possibility to use the chromatographic method also as HPLC-MS method, ammonium formate was selected for the buffer solution. Choice of ammonium formate also assured, that the mobile phase did not precipitate, when the amount of acetonitrile in the mobile phase was 90%. Afterwards, the influence of pH of formate buffer on the separation was investigated. Due to low buffer capacity of ammonium formate at pH values above 5, buffer solution was tested in the pH range 3.0–5.0. Separa-



Structure of pioglitazone and impurities P1, P2, P4 and P5

tion of compounds appeared too close and fast, when pH was lowered below 3.5. At pH values above 4.7, apparent deterioration of resolution between PG and the impurity P1 was observed. Therefore, a formate buffer pH of 4.1 was selected as optimal.

For the elution of all related substances, the gradient was started at 55% of acetonitrile and then the amount of acetonitrile was increased to 90%. Better separation and quicker reconditioning of the column were reached by using premixed eluents from the formate buffer and acetonitrile than by using "solely" acetonitrile as eluent A and ammonium formate buffer as eluent B.

It is generally known that in reversed-phase chromatography methanol has a lower elution strength than acetonitrile. From this reason, 75% methanol (methanol and water, 75 : 25, v/v) in purity test, respectively 68% methanol in assay test were used (sufficient solubility had to be assured) as final sample solvents without adverse effects on the peak shape. For chromatographic conditions of the optimized method see 3.5.1. The separation of PG and its impurities, characterized by retention time, capacity factor, tailing factor, resolution and number of theoretical plates, is presented in Table 1. The developed method is also suitable for evaluation of raw PG-HCl after synthesis and during purification steps. Well separated are all related substances originating in process of synthesis as shown in Fig. 1.

The placebo dos not interfere with PG and its impurities. All impurities are well separated. A chromatogram of a sample solution spiked with the impurities P1–5 is shown in Fig. 2.

To find potential degradation products arising from the specific combination of PG-HCl and excipients, a stress test with accelerated and long-term stability testing were performed. At stress test, the tablets and wetted tablets (amount of added water was 10% of the tablet mass) were heated for 24 h at 95 °C in tightly closed volumetric flasks. Accelerated stabilities run 6 months at 40 °C and 75% of relative humidity, long-term stabilities run 1 year at conditions corresponding to climatic zone II (25 °C and 60% of relative humidity). In all samples examined no degradation products were found and at the same time the amount of by-products already present in PG-HCl did not change.

Small changes in chromatographic conditions had no significant effect on the separation of PG and its impurities. The behavior of individual separated compounds, after small changes of chromatographic conditions, is shown in Table 2.

Reduction of the flow rate to approximately 0.6 ml/min and using of 0.01-M formate buffer instead of 0.05-M allows to use the method as HPLC-MS method. No significant change in separation with 0.01-M formate buffer was observed. An equivalent column for the separation is Li-

Table 1: Performance parameters of separated compounds in purity test

Compound	Retention time (min)	Capacity factor	Tailing factor	Resolution	Number of theoretical plates
Pioglitazone	6.1	2.4	1.01	–	18388
P1	7.8	3.3	1.14	7.2	19198
P2	14.8	7.2	1.10	27.0	40452
P3	16.2	8.0	1.08	5.5	72569
P5	17.5	8.7	1.09	6.3	105153
P4	18.7	9.4	1.11	6.1	154136

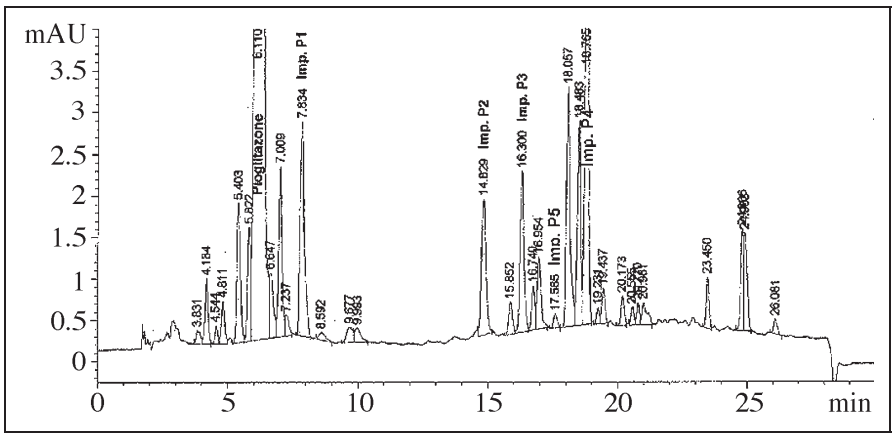


Fig 1:
Chromatogram of raw pioglitazone hydrochloride with content of 4% of impurities

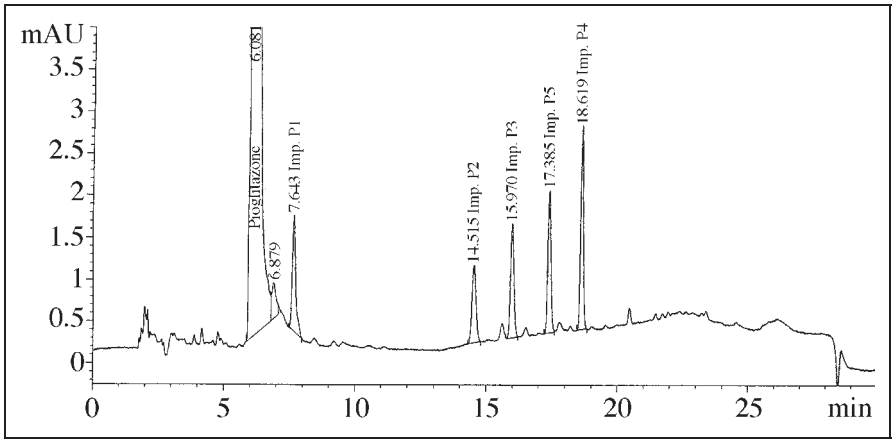


Fig. 2:
Chromatogram solution spiked with impurities P1-5

Table 2: Robustness of the purity method: influence of small changes in chromatographic conditions on seperation (comparison with optimized conditions)

pH	Retention time (min)						Resolution pioglitazone × P1
	Pioglitazone	P1	P2	P3	P5	P4	
3.90	5.7	7.4	13.3	15.2	17.0	18.0	7.5
4.10	6.1	7.8	14.8	16.2	17.5	18.7	7.2
4.30	6.3	8.0	15.5	16.8	17.8	19.1	7.2
Flow rate	Retention time (min)						Resolution pioglitazone × P1
	Pioglitazone	P1	P2	P3	P5	P4	
0.8 ml/min	7.6	9.7	16.9	18.2	19.7	20.5	7.6
1.0 ml/min	6.1	7.8	14.8	16.2	17.5	18.7	7.2
1.2 ml/min	5.1	6.5	12.9	14.7	15.9	17.4	6.9
Temperature	Retention time (min)						Resolution pioglitazone x P1
	Pioglitazone	P1	P2	P3	P5	P4	
25 °C	6.1	8.0	14.6	16.2	17.7	18.8	7.4
30 °C	6.1	7.8	14.8	16.2	17.5	18.7	7.2
35 °C	6.1	7.7	14.9	16.3	17.4	18.7	7.0
Gradient program	Retention time (min)						Resolution pioglitazone × P1
	Pioglitazone	P1	P2	P3	P5	P4	
gradient 1*	7.6	10.3	17.6	18.7	19.7	20.8	8.8
optimized gradient	6.1	7.8	14.8	16.2	17.5	18.7	7.2
gradient 2**	5.1	6.2	11.0	12.9	15.0	16.4	5.8
Colum LiChrospher 100 RP-18e, 5 µm, 250 × 4.0 mm ***	Retention time (min)						Resolution pioglitazone × P1
	Pioglitazone	P1	P2	P3	P5	P4	
	6.5	8.6	16.0	17.5	18.5	19.6	7.6

* Gradient 1

Time (min)	Eluent A (%)	Eluent B (%)	Comment
0–10	10	90	isocratically
10–20	10 → 80	90 → 20	linear gradient
20–25	80	20	isocratically
25–26	80 → 10	20 → 90	linear gradient
26–35	10	90	equilibration

** Gradient 1

Time (min)	Eluent A (%)	Eluent B (%)	Comment
0–10	30	70	isocratically
10–20	30 → 100	70 → 0	linear gradient
20–25	100	0	isocratically
25–26	100 → 30	0 → 20	linear gradient
26–35	30	70	equilibration

*** Flow on column LiChrospher was 0.8 ml/min (different column diameter was taken into account)

chrospher 100 RP-18e column, 5 µm, 250 × 4.0 mm; the separation on this column was very similar. Sample solutions were stable for 24 h after the preparation at laboratory temperature and without protection from light.

Two criteria for system suitability test were:

- Resolution between peaks of PG and impurity P1 should be at least 4.0.
- Relative standard deviation (RSD) of PG peak areas for 5 replicated injections of PG reference solution should not exceed 3%.

Acquired values of resolution, during method validation, were not lower than 6.5, acquired values of RSD were up to 0.5%.

Linearity of PG peak area/concentration dependence was verified within the concentration range 0.12–6.0 µg/ml of PG-HCl (0.02–1.0% of sample solution). The correlation coefficient r was found to be 0,99999 in this range. The equation of the calibration curve was: $y = 17.46x + 0.22$.

The limit of detection (LOD) was calculated according to:

$$LOD = \frac{3.3 \cdot \sigma}{S} \qquad (1)$$

where σ is noise peak-to-peak of baseline in the chromatogram of placebo solution, S is slope of the regression line acquired by measuring of the linearity in the test of purity (dependency peak height on concentration).

The limit of detection of PG-HCl was 41.7 ng/ml, which corresponds to 0.007% of sample solution. The limit of quantification of PG-HCl is triplicate of this value, i.e. 125 ng/ml (0.021% of sample solution).

Amount of single impurity (x) in % in evaluated tablets was calculated according to:

$$x = \frac{A_S \cdot c_R \cdot w_{TBL} \cdot 5 \cdot C \cdot RRF}{A_R \cdot w_S \cdot D} \qquad (2)$$

where A_S is peak area of evaluated impurity in the chromatogram of sample solution, A_R is peak area of PG in the chromatogram of reference solution, c_R is concentration of PG-HCl in the reference solution in µg/ml, w_S is weight of the powdered tablets in mg, w_{TBL} is average weight of 1 tablet in mg (~170 mg), C is correction of PG-HCl to PG (C = 0.9072), D is amount of PG in 1 tablet in mg (30 mg), RRF is relative response factor of evaluated impurity.

Table 3: Gradient program

Time (min)	Eluent A (%)	Eluent B (%)	Comment
0–10	20	80	isocratically
10–20	20 → 90	80 → 10	linear gradient
20–25	90	10	isocratically
25–26	90 → 20	10 →80	linear gradient
26–35	20	80	equilibration

Relative response factors (RRF) of isolated impurities with respect to PG under optimized chromatographic conditions, were: impurity P1: 0.91; impurity P4: 0.71; impurity P5: 1.00. The impurities P2 and P3 were not characterized by RRF. The concentration of compounds in the analyte used for determination of RRF was 1.2 μg/ml.

Six sample solutions (see experimental) were prepared and analyzed. Repeatability, calculated from values of impurities (impurities over limit of quantification), was found to be 2.3%.

By method of standard additions, the impurities P1, P4 and P5 were added to model samples (123 mg of placebo and 29.8 mg of reference substance PG-HCl) at levels 0.05%, 0.2% and 0.5% in sample solution. Three replicates for each level were prepared. The recoveries of impurities were calculated from the peak areas of impurities in the chromatograms of model sample solutions with reference to reference solution of impurities P1, P4, P5 (1.08 μg/ml). Found values of recoveries were for impurity P1 in the range 98.5%–103.2%, for impurity P5 in the range 96.8%–100.7%, and for impurity P4 in the range 98.6%–100.7%.

2.2. Assay method

As the assay method a shortened purity method was used. Injection volume and concentration of PG in the sample solution were lowered. Placebo does not interfere with PG.

Sample solutions were stable for 24 h after preparation at laboratory temperature and without protection from light.

Two criteria for system suitability test were:

- Resolution between peaks of PG and impurity P1 should be at least 4.0.
- Relative standard deviation of PG peak areas for 5 replicated injections of reference solution should not exceed 1.5%.

Acquired values of resolution, during method validation, were not lower than 6.5, acquired values of RSD were up to 0.2%.

Linearity was verified within the concentration range 36.7–123.5 μg/ml of PG-HCl (46.3–155.6% of the reference solution). Correlation coefficient r was found to be 0,99991 in this range. The equation of the calibration curve was: $y = 4.46x - 1.62$.

Repeatability, calculated from the results of six independently prepared and analyzed samples, was 0.67%.

Accuracy was tested by recovery of PG at levels of 80%, 100% and 120% of its declared content with 100% content of placebo. Three replicates for each level were prepared. Recoveries ranged from 99.19% to 99.74%. The 95% confidence interval was 99.30%–99.50%.

3. Experimental

3.1. Apparatus

HPLC separations were carried out on a system Hewlett Packard series 1100 equipped with a variable wavelength detector (HP GmbH, Waldbronn, Germany). Data acquisition and system control provided Chemstation software – revision A.08.03 from the same company. HPLC separations were performed on a C18 column Symmetry 5 μm, 250 × 4.6 mm (Waters, Milford, Massachusetts).

Other apparatuses were a scale Sartorius BP 211D with precision of 0.01 mg (Sartalex, Ústí nad Labem, Czech republic), a pH-meter Metrohm 691 (Metrohm, Herisau, Switzerland) and an ultrasonic bath with timer (Notus-Powersonic, Vráble, Slovakia).

3.2. Reagents and reference substances

Acetonitrile gradient grade, methanol HPLC grade and formic acid 98–100% extra pure were from Merck (Darmstadt, Germany), ammonium formate 97% was from Sigma-Aldrich (Steinheim, Germany). Source of purified water for HPLC separations was Milli-Q water system (Millipore S.A., Molsheim, France). As standard was used in-house reference substance pioglitazone hydrochloride (assay calculated on dried substance: 99.50%, loss on drying: 0.04%). Isolated impurities P1, P3, P4 and P5, arising during process of synthesis, were used for method validation. Structure of impurity P2 has not been completely confirmed yet.

3.3. Pharmaceutical preparation

Tablets were formulated from the PG-HCl and placebo (lactose, magnesium stearate, carboxymethylcellulose sodium, hydroxypropylcellulose). One tablet weighted 170 mg and contained 30.0 mg of pioglitazone (i.e. 33.07 mg of pioglitazone. HCl). Validation procedures were performed with 1 month old tablets.

3.4. Reference and sample solutions

3.4.1. Purity method

3.4.1.1. Sample solution

Into a 25-ml volumetric flask 153 mg of finely powdered tablets, were weighted 20 ml of methanol were added and the mixtur was sonicated for 10 min. The volumetric flask was filled up to the mark with methanol. The solution was centrifuged (5 min at 10000 rpm). 5.0 ml of the supernatant were diluted to 10.0 ml with 50% methanol (methanol and water, 50 : 50, v/v). The nominal concentration of pioglitazone-HCl in the sample solution was 0.6 mg/ml.

3.4.1.2. Stock solution

Pioglitazone-HCl reference substance (14.9 mg) was dissolved in methanol (sonicating 5 min) and diluted to 25.0 ml with the same solvent. 10.0 ml of this solution were diluted to 100.0 ml with 75% methanol.

3.4.1.3. Reference solution

1.0 ml of the stock solution of pioglitazone-HCl was diluted to 100.0 ml with 75% methanol.

3.4.1.4. Solutions for verifying linearity

11 solutions of pioglitazone-HCl within the concentration range of 0.12–6.0 μg/ml (0.02–1.0% of sample solution) were prepared from the stock solution by diluting with 75% methanol.

3.4.1.5. Solution for verifying resolution

4.0 mg of pioglitazone-HCl and 3.6 mg of impurity P1 were dissolved in methanol and then diluted to 25.0 ml with the same solvent. 1.0 ml of this solution was diluted to 2.0 ml with 50% methanol.

3.4.2. Assay method

3.4.2.1. Reference solution

Into a 50-ml volumetric flask 19.8 mg of pioglitazone-HCl reference substance, were weighed 40 ml of methanol were added and the mixture was sonicated for 5 min. The volumetric flask was filled up to the mark with methanol. 5.0 ml of this solution were diluted to 25.0 ml with 60% methanol.

3.4.2.2. Sample solution

Into a 50-ml volumetric flask 102 mg of finely powdered tablets were weighed, 40 ml of methanol were added and the mixture was sonicated for 10 min. The volumetric flask was filled up to the mark with methanol. The solution was centrifuged (5 min at 10000 rpm). 5.0 ml of supernatant were diluted to 25.0 ml with 60% methanol. The nominal concentration of pioglitazone-HCl in the sample solution was 0.08 mg/ml.

3.5. Chromatographic conditions

3.5.1. Purity method

Optimized chromatographic conditions were as follows: Injection volume 20 µl, flow rate 1.0 ml/min, temperature of column 30 °C, detection wavelength 266 nm and elution according to gradient program (see Table 3). Column Symmetry C18 5 µm, 250 × 4.6 mm (Waters) was used for separation.

Premixed eluents from ammonium formate buffer pH 4.1 and acetonitrile for better separation and quicker reconditioning of the column were used.

Eluent A: mixture of ammonium formate buffer pH 4.1 and acetonitrile (5 : 95, v/v).

Eluent B: mixture of ammonium formate buffer pH 4.1 and acetonitrile (55 : 45, v/v).

Ammonium formate buffer pH 4.1 (0.05-M) was prepared as follows: 3.15 g of ammonium formate was dissolved in 950 ml water, pH was adjusted to value 4.1 ± 0.1 with formic acid (diluted with water in ratio 1 : 5), buffer was diluted to 1000 ml with water and then was filtered through a 0.45-µm membrane filter.

3.5.2. Assay method

Optimized chromatographic conditions were as follows: Injection volume 5 µl, flow rate 1.0 ml/min, temperature of column 30 °C, detection wavelength 266 nm, time of analysis 9 min. Elution was isocratic, mobile phase was a mixture of ammonium formate buffer (0.05-M) pH 4.1 and acetonitrile (45 : 55, v/v). Column Symmetry C18 5 µm, 250 × 4.6 mm (Waters) was used for separation.

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