Development and Validation of a Stability-Indicating High Pressure Liquid Chromatography Method for Determination of Prostaglandin E1 and its Degradation Products in an Intracavernous Formulation

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Abstract

A new intracavernous formulation to overcome the failure of standard treatments of erectile dysfunction after radical prostatectomy was developed. This formulation contains prostaglandin E1 (pGE1) at 15 µg/ml in association with papaverin and urapidil at 15 mg/ml and 2.5 mg/ml respectively. Since pGE1 was the main compound to be subject to physico-chemical degradation, its degradation and the concomitant apparition of its degradation product prostaglandin A1 (pGA1) are correlated to the stability of the formulation. The low specific absorbance of PGE1 in the ultraviolet region, and the presence of high levels of papaverin and urapidil in the formulation were the principal difficulties to develop a new method for simultaneous determination of pGE1 and pGA1. Many compositions and pH of mobile phase were studied to find the best chromatographic conditions, and the chosen method is a sensitive, precise and accurate ramp reversed-phase high-performance liquid chromatographic (RP-HPLC) assay method. Ramp RP-HPLC separation was achieved on a Kromasil 5 C18 column (250×4.6 mm id, 5 µm particle size) using mobile phase composed of acetonitrile-pH 3 phosphate buffer (37 : 63%, v/v). The detection was performed at 205 and 230 nm for pGE1 and pGA1 respectively, using a multivave UV detector. The method was validated for specificity, linearity, precision, accuracy, robustness and sensibility. Limits of quantitation were about 3 µg/ml for pGE1 and 0.5 µg/ml for pGA1. The presence of urapidil and papaverin at high concentration did not interfere with determination of pGE1 and pGA1 in the formulation. The method developed which separates all the most degradation products formed under variety of conditions is sensitive, selective, linear, precise and accurate, and thus can be used to study the stability of the formulation.

Keywords: Formulation; Validation; Chromatography

Introduction

Erectile dysfunction is a well-known complication induced by abdominal and pelvic surgery. Radical treatment of a malignancy, vascular operations and transurethral resection can lead to the rise of these disorders. Some of the main common methods of management are penile implants, oral phosphodiesterase (PDE) inhibitors, vacuum devices, intraurethral prostaglandins (PG) and intracavernous injection of PG, papaverin or phenolamin [1-4]. Actual treatments of erectile dysfunction after prostatectomy are not completely efficient; some patients don’t respond or don’t tolerate intracavernous injection of high concentrations of PGE1 in monotherapy or in association with oral PDE inhibitors when they are not contra-indicated, thus leading to the development of “vasoactive cocktails” in intracavernous injection [5-7].

Most vasoactive cocktails reviewed in literature are also called “trimix” and are composed of PGE1, papaverin and phenolamin at variable concentrations [8,9].

Since phenolamin is not commercialized in France, it has been replaced by urapidil, another α-blocker.

The formulation studied in this work contains PGE1 at 15 µg/ml in association with papaverin and urapidil at 15 mg/ml and 2 mg/ml respectively; it was prepared from injectable commercial solutions of the different active substances and conditioned in syringes of 1 ml.

Since PGE1 is the main constituent subject to undergo physico-chemical degradation [2,10], following its degradation and concomitant apparition of its degradation product PGA1 enables to evaluate the stability of the formulation [11].

To study the stability of this mixture, it is necessary to have analytical methods allowing the separation of the numerous products and in particular which can measure PGE1, which has a low UV absorption. Various HPLC methods have already been described in the literature for determination of PGE1 by HPLC [12-15] but they are not used for determination in Trimix.

The aim of this work was to develop and validate according to the ICH description, a stability indicating RP-HPLC method that allows a simultaneous determination of PGE1 and its degradation product PGA1 in presence of urapidil and papaverin.

Experimental

Materials

All reagents used were of analytical grade. PGE1, PGA1, papaverin chlorhydrate, urapidil chlorhydrate and acetoniitrile were purchased from Sigma Aldrich, absolute ethanol used for the dissolution of...
prostaglandins was from VWR Prolabo, KH₂PO₄ and orthophosphoric acid were from Merk and water was from Versylene.

Prostine VR® (Alprostadyl 0.5 mg/ml injectable solution) from Pfizer, Papavérine SRB® (papaverin 40 mg/ml injectable solution) from SRB laboratories and Eupressyl® (urapidil 5 mg/ml injectable solution) from NYCOMED were used to prepare Trimix formulation.

Instrumentation

The development and validation work were carried out on a chromatographic system consisting of a Dionex Ultimate 3000 system, equipped with a variable wavelength UV-Vis detector model, a quaternary gradient pump equipped with a solvent programmer and a rheodyne model injector with a 100 µl sample loop. pH values were measured with a Consort P901 pH meter using a Fisher scientific pH glass electrode.

Prepared solutions

Stock standard solutions: For the preparation of the stock solutions, 1 mg of PGE₁ and PGA₁ were diluted with 2 ml of absolute ethanol leading to initial solutions of PGE₁ and PGA₁ of 500 µg/ml. Vials of these solutions were stored at -20°C for 3 months.

Working standard solutions: Working solutions were prepared from stock solutions by diluting an adequate volume in NaCl 0.9%. Final concentrations were 100 µg/ml and 10 µg/ml for PGE₁ and PGA₁ respectively. These solutions were not kept after use.

A blank solution was prepared from the injectable solutions of Papavérine SRB® 40 mg/ml and Eupressyl® 50 mg/10 ml. Concentrations of papaverin and urapidil in the blank were the same that in the final formulation.

In a second time, to reconstitute the solution, adequate volumes of work solution of PGE₁ were added into the blank solution.

Stock solution of PGE₁ (500 µg/ml) was diluted to obtain a working standard solution (100 µg/ml). Five calibration solutions over the range of the theoretical concentration (15 µg/ml) were prepared from this working solution, their concentrations were 12; 13.5; 15; 16.5 and 18 µg/ml corresponding to 80; 90; 100; 110 and 120% respectively of the nominal concentration of PGE₁.

Stock solution of PGA₁ (500 µg/ml) was diluted to obtain a standard work solution (10 µg/ml). Five calibration solutions were prepared from this working solution, their concentrations were 356.25 ng/ml; 712.5 ng/ml; 1.425 µg/ml; 2.85 µg/ml and 4.725 µg/ml corresponding to 2.5; 5; 10; 20 and 40% of PGE₁ degradation respectively, i.e. 25; 50; 100; 200; and 400% of the concentration limit of PGA₁ tolerated in the formulation.

Validation standard solutions of PGE₁ and PGA₁ were prepared in the following way: adequate volumes of working solution of PGE₁ (100 µg/ml) were added in synthetic mixture of all other components to obtain the same concentrations of PGE₁ in the calibration standard solutions.

Adequate volumes of working solution of PGA₁ (10 µg/ml) were added in synthetic mixture of all other components to obtain the same concentrations of PGA₁ as in the calibration standard solutions.

Chromatographic conditions

The separation was performed on a Kromasil C18 column (250×4.6 mm; 5 µm particles) with a (63:37 v/v) mixture of phosphate buffer (KH₂PO₄, 0.02 M, pH 3) and acetonitrile as mobile phase. The injection volume was 100 µl. The column was thermostated at 25°C. The mobile phase flow rate was variable: 0-15 min and 25-40 min: 1 ml/min; 15-25 min: 1.5 ml/min. Several detection wavelengths were used (205 nm, 230 nm and 280 nm) for detection of PGE₁, PGA₁ and PGB₁, which is a secondary degradation product of PGE₁.

Procedures for the method validation

Validation criteria were the specificity, precision, linearity, concentration range, accuracy and quantification limit.

Specificity: Specificity was demonstrated by examining potential interferences between formulation components, PGE₁ and its degradation product PGA₁, Papaverin and Urapidil. Specificity was established through study of resolution factors of the drug peak from the nearest resolving peak.

Precision: Six injections of the theoretical concentration of 15 µg/ml for PGE₁ and 1.425 µg/ml for PGA₁ (corresponding to 10% degradation of PGE₁) were performed on the same day and relative standard deviation (RSD) was calculated to determine repeatability or intra-day precision. This assay was repeated for 2 days in order to determine intermediate precision or inter-day precision.

Linearity and range: Linearity was determined by injecting calibration standard solutions (n=5) and standard validation solutions (n=5) for three days.

Accuracy: The study of accuracy was coupled to the linearity by calculating the recovery between theoretical concentrations of validation standard solutions and those found using calibration curve obtained from calibration standard solutions.

Limit of detection (LOD) and limit of quantitation: LOD and LOQ were calculated experimentally. LOD was defined as the concentration that yields a signal to noise ratio of 3.

The limit of quantitation was calculated to be the lowest concentration that could be measure with a signal to noise ratio of 10.

Degradation studies: Forced degradation studies were performed on the stock solution of PGE₁ to establish its inherent stability characteristics in order to demonstrate selectivity and stability indicating capability of the proposed method. The standard substances were exposed to:

- Acidic (0.1N HCl, room temperature, 3 hours), -Alkaline (0.1N NaOH, room temperature, 3 hours), -Strong oxidizing (3% H₂O₂, 80°C, 1 h)
- Thermal degradation conditions (80°C, 1 day)

All the exposed samples were then analyzed by the proposed method.

Results and Discussion

HPLC method development and optimization

The low specific absorbance in the UV zone of PGE₁ and the presence of high levels of papaverin and urapidil in the formulation are the principal difficulties to develop a HPLC-UV method for simultaneous determination of PGE₁ and PGA₁.

Although PGE₁ presents a low specific absorbance in the UV zone, UV detection mode was preferred to other detection modes cited in
The high levels of papaverin and urapidil in the preparation engendered very wide peaks saturating the detector, the resolution of PGE1 and PGA1 depended largely on the wideness of papaverin-urapidil peak. The chromatographic conditions were optimized in order to achieve good resolution, symmetrical peak shapes and short analysis time.

Selection of wavelength: The UV detector used allows setting four different wavelengths at the same time, wavelengths selected were 205 nm for detection of PGE1 and 230 nm for detection of PGA1. PGA1 and PGB1 have similar chromatographic properties; they will be eluted at the same time. On the other hand, they have very different spectral properties so we chose a third wavelength for detection of PGB1, 278 nm.

Development of the optimum mobile phase: The choice of composition and pH of mobile phase represents an important point during the development of the chromatographic methods. The chosen organic solvent was acetonitrile because its cut-off (190 nm) is compatible with detection at 205 nm, different proportions of acetonitrile and phosphate buffer (KH₂PO₄ 0.02 M) at different pH have been tested, only a pH under 3.5 and a proportion of acetonitrile under 40% allowed a good resolution between the peaks of papaverin and urapidil which were co-eluted and the peak of PGE1. The proportion of 37% acetonitrile at pH 3 lead to a good separation of urapidil-papaverin and PGE1 and the "shortest" retention time possible for PGA1, which was about 39 min.

Optimisation of flow rate: In order to shorten retention time of PGA1, a ramp of flow rate has been used: from 0 to 15 min and from 25 to 40 min the flow rate was 1 ml/min and from 15 to 25 min it was 1.5 ml/min.

Within these conditions, retention time of PGE1 and PGA1 were about 11 min and 29 min respectively (Figure 1).

Method validation

Specificity: The method was specific enough to have a good separation between PGE1 and PGA1 from one hand (Figure 2) and between PGE1 and high concentrations of urapidil and papaverin in the other hand (Figure 3).

The good separation between the peak of PGE1, the peak of PGA1 and the other compounds of the formulation confers a good specificity to the method.

Precision: The %RSD values for intra-day precision and intermediate precision were <2% (1.188% and 1.139% for intra-day precision and 0.608% and 0.744% for intermediate precision for PGE1 and PGA1 respectively), which confirms that the method is sufficiently precise (Tables 1 and 2).
Linearity: A good linear relationship was obtained between concentration and peak area for both PGE1 and PGA1, showing that the method is linear with a correlation coefficient greater than 99% (99.77% and 99.99% for PGE1 and PGA1 respectively).

Accuracy: Percentages of recovery were calculated from differences between theoretical concentrations and those calculated using calibration curve. As shown from data in tables 3 and 4, excellent recoveries were found for both PGE1 and PGA1 with CI95% ranging between [0.9958; 1.0053] and [0.9879; 1.0090] respectively.

Limit of detection (LOD) and limit of quantitation (LOQ): LOD calculated was 94, 43 ng/ml. LOQ calculated was 314 ng/ml. This value is much lower than 356 ng/ml, lower concentration (was) tested.
during the validation of the method. The limit of quantification of PGA1 retained for the method is 356 ng / ml. At this concentration, it was possible to measure pGA1 by the method with accuracy.

Degradation studies: The resulting chromatogram of PGE1 and its major degradation products obtained under stressed conditions is shown in figure 4. Our results are in accordance with previously studies which described degradation of PGE1 [10]. Degradation of PGE1 was observed under acid as well as alkaline conditions but the degradation which described degradation of PGE1 [10]. Degradation of PGE1 was strongly accelerated with the increase of pH. We also observed degradation of PGE1 and increase of PGA1 in oxidative conditions and in thermal conditions. It indicates that the method is optimized to separate PGE1 and its major degradation products (PGA1) formed under various conditions.

**Figures**

- **Figure 4**: Degradation Studies: resolution was optimized to separate major degradation products. Resolution was checked for all stress conditions tested. The resulting chromatogram with degradation products obtained under stressed conditions is shown in this figure. Chromatogram 1 (black): NaOH 0.1N 24 hours, Chromatogram 2 (blue): 80°C 24 hours, Chromatogram 3 (pink): HCl 0.1N 24 hours, Chromatogram 4 (brown): H₂O₂ 3% 24 hours, Chromatogram 5 (green): T0.

### Table 1: Intra-day precision of PGE1 quantitation.

<table>
<thead>
<tr>
<th>PGE1</th>
<th>Actual concentration (µg/ml)</th>
<th>AUC (mAU*min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>5.286</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>5.638</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>5.647</td>
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<td>4</td>
<td>15</td>
<td>5.640</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>5.694</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>5.533</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>5.6143</td>
</tr>
<tr>
<td>S.D</td>
<td></td>
<td>0.067</td>
</tr>
<tr>
<td>R.S.D</td>
<td></td>
<td>1.188</td>
</tr>
</tbody>
</table>

### Table 2: Intra-day precision of PGA1 quantitation.

<table>
<thead>
<tr>
<th>PGA1</th>
<th>Actual concentration (µg/ml)</th>
<th>AUC (mAU*min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.425</td>
<td>1.7328</td>
</tr>
<tr>
<td>2</td>
<td>1.425</td>
<td>1.7466</td>
</tr>
<tr>
<td>3</td>
<td>1.425</td>
<td>1.7362</td>
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<td>4</td>
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<td>1.7600</td>
</tr>
<tr>
<td>5</td>
<td>1.425</td>
<td>1.7396</td>
</tr>
<tr>
<td>6</td>
<td>1.425</td>
<td>1.7068</td>
</tr>
<tr>
<td>Average</td>
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<td>1.7360</td>
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<tr>
<td>S.D</td>
<td></td>
<td>0.020</td>
</tr>
<tr>
<td>R.S.D</td>
<td></td>
<td>1.139</td>
</tr>
</tbody>
</table>

### Table 3: % of recoveries for PGE1.

<table>
<thead>
<tr>
<th>Theoretical concentration PGE1 (µg/ml)</th>
<th>Calculated concentration (µg/ml) ± SD</th>
<th>Recovery (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>11.976 ± 0.081</td>
<td>99.8 ± 0.3</td>
</tr>
<tr>
<td>13.5</td>
<td>13.495 ± 0.072</td>
<td>99.96 ± 0.3</td>
</tr>
<tr>
<td>15</td>
<td>15.050 ± 0.092</td>
<td>100.33 ± 0.3</td>
</tr>
<tr>
<td>16.5</td>
<td>16.531 ± 0.155</td>
<td>100.19 ± 0.3</td>
</tr>
<tr>
<td>18</td>
<td>17.976 ± 0.086</td>
<td>99.86 ± 0.3</td>
</tr>
</tbody>
</table>

### Table 4: % of recoveries for PGA1.

<table>
<thead>
<tr>
<th>Theoretical concentration PGA1 (µg/ml)</th>
<th>Calculated concentration (µg/ml) ± SD</th>
<th>Recovery (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35625</td>
<td>0.354 ± 0.017</td>
<td>99.37 ± 0.3</td>
</tr>
<tr>
<td>0.7125</td>
<td>0.713 ± 0.010</td>
<td>100.07 ± 0.3</td>
</tr>
<tr>
<td>1.425</td>
<td>1.426 ± 0.005</td>
<td>100.07 ± 0.3</td>
</tr>
<tr>
<td>2.85</td>
<td>2.848 ± 0.029</td>
<td>99.93 ± 0.3</td>
</tr>
<tr>
<td>4.275</td>
<td>4.271 ± 0.010</td>
<td>99.91 ± 0.3</td>
</tr>
</tbody>
</table>

S.D = Standard Deviation
R.S.D = Relative Standard Deviation

### References


