Quantification of Cyclofenil Using High-Performance Liquid Chromatography with Fluorimetric Detection and Photochemical Derivatization

Abstract
Cyclofenil is an anti-estrogen employed to treat ovarian disorders and it is listed by the World Anti-Doping Agency as doping for male athletes as its use to increase the concentration of testosterone is illicit. In this work, a high-performance liquid chromatography method was developed for the determination of cyclofenil by measuring the intense fluorescence from a stable photochemical derivative, generated by UV (after 40 min) enabling a single sharp chromatographic peak (retention time of 2.5 min). Limit of detection of $6.7 \times 10^{-8}$ mol L$^{-1}$ (24 ng mL$^{-1}$) was achieved. The combined uncertainty (considering contribution of diverse sources of uncertainties) was 16% at the $1.2 \times 10^{-5}$ mol L$^{-1}$ analyte concentration level. Recoveries achieved in pharmaceutical formulations ranged from 94% and 105%. Studies using the method indicated the homogeneity of cyclofenil in the analyzed tablets. The drug was also found to be stable in pharmaceutical tablets exposed to room-ambient light.

Keywords: Cyclofenil; Photochemical derivatization; High performance liquid chromatography; Drug stability

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Introduction

Photochemical derivatization consists in pre-treat a sample using ultra-violet (UV) photons to transform the original analyte in new substances with properties that bring improvements in analytical performance. Reactions induced by UV photons promote the target molecule to a more energetic level that causes bonding disruptions, leading to products with a more rigid molecular structure, thus with higher fluorescent quantum efficiencies [1,2]. In photochemical derivatization the use of costly and toxic chemical reagents is avoided and clean and controlled reaction conditions can be achieved [2].

Cyclofenil (Figure 1A) is an antiestrogen drug that affects the synthesis and secretion of follicle stimulating hormone (FSH) and the luteinizing hormone. In women, FSH stimulates ovulation and estradiol hormone production during the first half of menstruation [3]. In males, the use of cyclofenil increases testosterone in blood, causing the growth of muscle, increased strength and aesthetics [4], thus, the use of this drug, classified as hormone modulator by the World Anti-Doping Agency (WADA), is considered as doping [5].

Some analytical methods are described in the literature for the determination of cyclofenil. Hassan et al. reacted cyclofenil with H₂SO₄ and measured the absorbance of the reaction product (non-identified) at 272 nm [6]. This absorption spectrophotometric method was applied to pharmaceutical formulation with analyte recoveries of 101.06 ± 0.78%. Crucús used flame ionization gas chromatography to determine cyclofenil after its extraction from bovine muscle tissue [7]. Analyte recovery of 95% has been reported with detection capability estimated to be 0.02 µg g⁻¹. Pacheco et al. used square-wave voltammetry to determine cyclofenil, previously submitted to UV [8]. A single current peak was produced at the working mercury electrode. The reported limit of detection (LOD) was 1.5 × 10⁻⁸ mol L⁻¹, with linear range up to 6.0 × 10⁻⁷ mol L⁻¹. Pharmaceutical formulations and analyte fortified urine samples were analyzed with recoveries ranging from 93.6 to 106.5%.

Gartner et al. studied the synthesis of hydroxylated metabolites of cyclofenil and characterized their chemical structure using gas chromatography with mass spectrometric detection (GC-MS) with electron spray ionization [9]. Authors identified, after sialanization, metabolites hydroxylated at the 2-, 3- or 4-position of the aliphatic ring in addition to the phenolic functions on the aromatic rings (converted from the original ester groups of the molecule) in reference male urine samples provided by WADA. Pacheco et al. exposed cyclofenil (in basic solution) to UV in order to produce derivatives with intense fluorescence, enabling the spectrofluorimetric indirect determination of the analyte [2]. Data from laser desorption/ionization time-of-flight MS and GC-MS confirmed two cyclofenil phenolic derivatives with m/z values 322 and 288 (Figure 1B) that produce fluorescence bands with the same spectral range (250/410 nm). A photochemical mechanism was proposed based on the conversion of ester groups into hydroxyl groups, producing derivatives with higher structural rigidity thus higher quantum fluorescence efficiencies. The spectrofluorimetric method enabled a LOD value of 11 nmol L⁻¹ with linear range up to 8 × 10⁻⁷ mol L⁻¹ and analyte recovery of 98.3 ± 3.9% in pharmaceutical formulations. Myung et al. detected cyclofenil metabolites in urine using GC-MS and high performance liquid chromatography with tandem mass spectrometric detection (HPLC MS/MS) [10]. They detected metabolites with the hydroxylated aliphatic ring along with the one with non-hydroxylated aliphatic ring (in both cases with the two phenolic groups). In addition the glucuronide conjugates of these metabolites were found. The original cyclofenil was not found in urine and authors did not report any analytical figure of merit to indicate detection capability and precision in analysis. Brabanter et al. used CG-MS (triple quadrupole) to screen doping substances in urine, including cyclofenil, which was indirectly quantified by its metabolite 4-hydroxycyclofenil [11]. The reported LOD was 2.5 ng mL⁻¹ but indicating a minimum required performance limit for quality assurance of 50 ng mL⁻¹.

Despite the fact that a few analytical methods for the determination of cyclofenil are available in literature, there is no quantitative approach based on HPLC. Besides, for the existent chromatographic methods, there is a lack of information on validation and no study on the impact of the homogeneity of sample and the stability of cyclofenil to ambient light on the analytical results has been addressed. The present work aims to provide a sensitive HPLC method for cyclofenil based on the fluorimetric detection of a stable photoderivative. Proper validation, including with a thoroughly uncertainty information and the evaluation of the homogeneity and stability of a commercial cyclofenil based drug are presented.

Experimental

Instrumentation

The HPLC method was developed on a Waters Breeze 1525 HPLC system (USA) consisting on a binary pump, a thermostatic unit, a model 2475 fluorescence detector, a Rheodyne manual injector (with a 20 µL sampling loop) and a Breeze 3.30 (2002) software. Separations were made using a C18 XTerra column (4.6 × 150 mm, 5 µm particle size). The laboratory made a photochemical reactor consisted of six sterilization mercury lamps (6 W each) placed inside a cylindrical PVC cabinet (200 mm of diameter and 290 mm of depth), which enabled uniform radiation over the samples (accommodated in 20 mL quartz tubes placed in the center part of the reactor). A small fan was placed at the back of the reactor in order to keep its internal temperature stable (temperature did not surpass 30°C).

Reagents and chemicals

Ultrapure water was obtained from the Milli-Q gradient A10 ultra-purifier (Millipore, USA). The cyclofenil standard was from Sigma-Aldrich (EUA). Sodium hydroxide and sodium tetraborate were obtained from Merck (Germany). HPLC grade methanol was obtained from Tedia (USA). One brand of commercial pharmaceutical preparation containing cyclofenil as the active principle (200 mg per tablet) was purchased in local drugstores.

Preparation of standard and sample solutions

Cyclofenil stock solution (5 × 10⁻⁴ mol L⁻¹) was prepared by dissolving a known amount of the standard in methanol. For medicine samples, the solutions (containing about 1 × 10⁻⁵ mol L⁻¹ of cyclofenil) were prepared by dissolving a known amount of
the drug (aliquots of mass previously pulverized and taken from one tablet or from a pool of tablets) in methanol. This solution was loaded on a C18 solid phase extraction (SPE) column in order to retain cyclofenil and enable the washing of the sample with water to eliminate water soluble tablet components. Cyclofenil was eluted from the cartridge with methanol with recoveries for the analyte above 99%. The subsequent dilutions for drug samples and working standards solutions of cyclofenil were made using methanol/borate buffer (pH 10.5; 10 mmol L-1) 60/40% V/V mixture. All solutions were stored under refrigeration and protected from ambient light.

Photoderivatization procedure

The photoderivatization of cyclofenil was made by exposing cyclofenil solutions, previously prepared by dissolving amounts of the stock solution (or sample solution) in methanol/borate buffer (pH 10.5; 10 mmol L-1) 60/40% V/V. These solutions (5 mL) were transferred to 10 mL quartz tubes, placed in the reactor and exposed to the UV light (60 min) in order to maximize production of the photoderivative, with retention time (t_R) at 2.5 min, used to indirectly detect cyclofenil. After removed from the reactor, the solutions were kept in the dark until analyzed (no more than a few hours after preparation).

Chromatographic analysis

Mobile phase solvents were previously filtered through a 0.2 µm borosilicate glass microfiber membrane. The solvents were then degassed (off-line) in a 9 L ultrasonic bath (Unique, Brazil). Chromatographic runs were made under isocratic conditions with methanol/borate buffer (pH 10.0; 10 mmol L-1) 60/40% V/V at 0.8 mL min⁻¹ flow rate. Sample volume injection was 20 µL and the column was kept at 35°C. Under irradiation, cyclofenil gradually generates what was called cyclofenil photodervative I (CyPh I with t_R = 9.4 min), which, in time, are transformed into the photodervative II (CyPh II with t_R = 4.5 min), that generate a final and more stable photodervative III (CyPh III with t_R = 2.5 min). Fluorescence signal was monitored at 278/308 nm in the solution.

Homogeneity and stability studies

Homogeneity study was made by comparing the analytical signal produced by individual tablets (ten pulverized tablets) and the signal from the pool of these pulverized tablets. A specific portion of each pulverized tablet and from the pool of tablets were placed in methanol to dissolve cyclofenil. The mixture was centrifuged and a volume of the supernatant was collected and diluted to the final solution in a methanol/borate buffer (pH 10.5; 10 mmol L-1) 60/40% V/V composition. Solutions were exposed to UV during 60 min and introduced into the HPLC system to measure the fluorescence from CyPh III.

Stability study was made with the pool of ten pulverized tablets divided in two portions. The fluorescence from CyPh III was measured right after solution preparation. The other portion was divided in two others to perform a long-term study: one stored under exposition to ambient light and the other kept in the dark (control) both during 25 days before dissolution and analyzed by HPLC. No photoderivatization procedure was performed in these samples since it was intended to verify the natural formation of cyclofenil derivatives under ambient light.

Results and Discussion

Photochemical derivatization and chromatographic conditions

Pacheco et al. have studied the production of cyclofenil derivatives after exposition to UV radiation [2]. The reaction was more efficient at basic conditions (in 90% V/V methanolic medium containing 10% V/V of aqueous Britton-Robinson (pH 10.8 buffer) with the increasing of fluorescence forming two plateaus, in function of the UV exposition time. The two identified cyclofenil phenolic derivatives produce fluorescence at the same spectral range (250/410 nm). The stepwise increasing of signal indicated the improvement of the fluorescence quantum yield due firstly to the formation of a more rigid derivative after conversion of one terminal ester groups into hydroxyl (mass-to-charge ratio equals 322 Da) followed by the formation of a second even more rigid derivative after the conversion of the other terminal ester groups into hydroxyl (mass-to-charge ratio equals 288 Da) [2]. They also described that the reaction efficiency changed in function of the pH and the water content in the solution.

In order to adapt the conditions to produce the cyclofenil photodervatives to liquid chromatographic analysis, the aqueous proportion of the solvent system, used to prepare the cyclofenil solution, was increased to 40% V/V, keeping at least 60% V/V of methanol to ensure total solubilization of cyclofenil. The pH of this solution was adjusted using borate buffer (pH 10.0; 10 mmol L⁻¹). These cyclofenil solutions (2 × 10⁻⁵ mol L⁻¹) were exposed to UV during 10 min to be introduced into the HPLC system. Three main peaks with baseline separation (t_R at 2.5; 4.5 and 9.4 min) were detected using a compromise fluorescence excitation/emission pair (278/308 nm) as seen in Figure 2. The chromatogram obtained from the blank solution can be seen in Figure 3B (chromatogram a). Mobile phase of the same composition of the analyte solution, methanol/borate buffer (pH 10.0; 10 mmol L⁻¹) 60/40 V/V, was used at a flow rate of 0.8 mL min⁻¹. Cyclofenil originally presents weak fluorescence in solution and it was not detected.

It is interesting to notice that three photodervatives of cyclofenil were detected instead of the main two identified by Pacheco et al. [2]. However, it is important to point out that the mentioned authors made the first measurement after 20 min of UV exposition, when the first formed photodervative was probably totally converted into the other ones. The water proportion of the solution exposed to the UV was also different, which may affect the derivatization process. In addition, the design and the intern temperature of the reactors were completely different, which may affect the rate of the reaction as the one used before [2] reached 60°C, under operation, while the temperature of the more compact reactor used in the present work was designed not to surpass 30°C.

As the fluorescence of the solution was monitored (using the HPLC system with the compromise pair at 308/378 nm) over the UV exposition time, the profile trends for the different photodervatives (Figure 3A) were constructed. The data was
conditions in Table 1. Methanol/borate buffer (pH 10.5; 10 mmol L\(^{-1}\)) was used in the solution submitted to UV irradiation. It can be seen that the use of borate buffer (pH 10.5) produced a more efficient use of the HPLC system to be monitored at the best fluorescence pair for the photodervative (328/374 nm). As the column temperature was changed from 25°C to 40°C, no significant effects were in terms of neither retention time (less than 0.2 min) nor in peak broadness. A further experiment was made in order to evaluate the effect of changing the pH (from 9.0 to 11.5) of the buffer used in the solution submitted to UV irradiation. It can be seen that the use of borate buffer (pH 10.5) produced a more efficient conversion of cyclofenil into CyPh III as seen in Figure 5.

Peak symmetry of 0.9 was achieved. Considering the column length of 15 cm was used and according to data the number of theoretical plates was 4.7 \(\times 10^2\) and plate height of 3.1 \(\times 10^{-2}\) cm. The experimental conditions chosen to enable the indirect determination of cyclofenil by means of the stable CyPh III photodervative are shown in Table 1.

### Analytical characteristics

Considering the signal of the chromatographic CyPh III peak, the linear response was described by the \(Y = (5.7 \pm 0.2) \times \)
The limit of detection (LOD) and the limit of quantification (LOQ) were respectively $6.7 \times 10^{-4}$ mol L$^{-1}$ (24 ng mL$^{-1}$) and $7.3 \times 10^{-7}$ mol L$^{-1}$ (79 ng mL$^{-1}$) calculated considering the concentration equivalent to a signal equivalent to 3$\sigma$ (for LOD) and 10$\sigma$ (for LOQ), where $\sigma$ is the standard deviation 10 signal measurements. The homogeneity of variances were also used to evaluate linearity, using the Fisher-Snedecor and the t-Student statistical tests, which indicated good fit to the linear model, verified by a random distribution of residues. Analysis of variance showed significant regression ($p < 0.001$) and the t-Student statistical tests, which indicated good fit to the linear model ($p > 0.05$), confirming homoscedastic behavior.

Intra-day ($u$) and inter-day ($u_b$) precisions were calculated from experiments performed on ten consecutive days. From the $F$ values obtained, it was possible to confirm that there was no significant difference among variances. The average result of the uncertainty of the analytical curve ($u_{cur}$) besides $u$ and $u_b$ can be seen in the proposed cause-and-effect diagram (Figure 6).

The $u_{cur}$ was calculated from the parameters of the analytical curve using cyclofenil concentrations within the linear response range. The standard deviations for both the angular ($m$) and the linear coefficient ($b$) of the curve were calculated in order to get their respective uncertainties, $u_m$ and $u_b$. From these, the $u_{cur}$ was calculated using the expression $u_{cur} = \left((c_{\sigma_b}^2 \times s_b^2) + (c_{\sigma_m}^2 \times s_m^2) + (2 \times c_{\sigma_b} \times c_{\sigma_m} \times u_m \times u_b \times r)\right)^{1/2}$, where, $c_{\sigma_b}$ and $c_{\sigma_m}$ are sensitivity coefficients and $r$ is the correlation coefficient. The sensitivity coefficients were used to standardize the units of the uncertainties, allowing the quadratic summation to be performed. Their values were obtained from $c_{\sigma_b} = -1/m$ and $c_{\sigma_m} = (p-b)/m^2$ that were achieved from the partial derivative of the calibration curve equation in respect to the linear and angular coefficients. The $u_{sol}$ value was calculated from the expanded uncertainties from the balance, $U_{bal}$, and from the volumetric apparatures, in this case, the expanded uncertainties from the volume microliter pipette ($V_{\text{fl}}$) and from the volumetric flask ($V_{\text{f}}$) used in the preparation of the analyte solution, respectively indicated as $U_{sol}$ and $U_{fl}$. The uncertainty was obtained from $u_{sol} = U_{fl} \cdot k$ and from $u_{sol} = U_{fl} \cdot k$ and from $u_v = U_{fl} / k$ where $U_{fl}$ values were obtained from calibration certificates and $k = 2$ (the chosen coverage factor, 95.4%). The $u_{sol}$ value was calculated as the square root of the quadratic summation of the uncertainty values of the balance ($u_{bal}$) and of the volumetric apparatures ($u_{fl}$ and $u_{sol}$), multiplied by the dilution factor uncertainty ($u_d$), calculated from: $u_d = [(1/V_{sol}) \cdot u_{sol}^2 + (V_{sol}/V_{f}) \cdot u_{sol}^2]^{1/2}$, where $1/V_{sol}$ and $V_{sol}/V_{f}^2$ are sensitivity coefficients [12].

The combined uncertainty ($U$) was obtained by performing the quadratic addition of these four contributions: $U = (u_d^2 + u_{sol}^2 + u_{cur}^2 + u_b^2)^{1/2}$. The expanded uncertainty ($U$) was calculated


Table 1: Selected conditions for the HPLC method based for the determination of cyclofenil using its stable photochemical derivative.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Chosen condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borate buffer concentration</td>
<td>10 mmol L$^{-1}$</td>
</tr>
<tr>
<td>pH</td>
<td>10 (mobile phase) and 10.5 (reaction solution)</td>
</tr>
<tr>
<td>Temperature</td>
<td>35 °C</td>
</tr>
<tr>
<td>UV exposure time</td>
<td>60 min</td>
</tr>
<tr>
<td>Sample introduced volume</td>
<td>20 µL</td>
</tr>
<tr>
<td>Elution</td>
<td>Isocratic</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>60/40% V/V methanol/ borate buffer</td>
</tr>
<tr>
<td>Mobile phase flow rate</td>
<td>0.8 mL min$^{-1}$</td>
</tr>
<tr>
<td>Detection ($\lambda_{sol}/\lambda_{fl}$)</td>
<td>328/374 nm</td>
</tr>
</tbody>
</table>

Table 2: Uncertainty values (expressed in terms of concentration of cyclofenil) for grouped uncertainty sources at four different cyclofenil concentrations within the linear fluorimetric response.

<table>
<thead>
<tr>
<th>Type of uncertainty</th>
<th>Studied concentration of cyclofenil (mol L$^{-1}$)</th>
<th>Uncertainty values expressed as equivalent cyclofenil concentrations (mol L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeatability ($u_r$)</td>
<td>$9.0 \times 10^{-4}$</td>
<td>$1.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>Intermediate precision ($u_i$)</td>
<td>$1.5 \times 10^{-7}$</td>
<td>$1.5 \times 10^{-7}$</td>
</tr>
<tr>
<td>Preparation of solutions ($u_p$)</td>
<td>$7.3 \times 10^{-7}$</td>
<td>$7.3 \times 10^{-7}$</td>
</tr>
<tr>
<td>Analytical curve ($u_{cur}$)</td>
<td>$6.7 \times 10^{-7}$</td>
<td>$6.7 \times 10^{-7}$</td>
</tr>
<tr>
<td>Combined uncertainty ($u_{combined}$)</td>
<td>$1.0 \times 10^{-6}$</td>
<td>$1.0 \times 10^{-6}$</td>
</tr>
<tr>
<td>Combined uncertainty (%)</td>
<td>34%</td>
<td>16%</td>
</tr>
</tbody>
</table>
by multiplying $u_c$ by a coverage value ($k$), in this case 2, in order to express the uncertainty at a confidence level of 95% (Table 2). The combined uncertainty equivalent to 1.2 $\times$ 10^{-3} mol L^{-1} of cyclofenil (the average concentration of the analytical curve) was 8%, which in terms of analyte concentration is equivalent to 9.6 $\times$ 10^{-7} mol L^{-1}. At the lowest concentration (3.0 $\times$ 10^{-6} mol L^{-1}) of the curve, the combined uncertainty is obviously greater (34%). Uncertainty results considering four different analyte concentrations are indicated in Table 2.

**Application of the method**

One pharmaceutical formulation (containing the nominal value of 200 mg of the cyclofenil per tablet) was analyzed. Subsamples from a pool of ten pulverized tablets were taken and the recovered results were between 95 and 104% in the four concentration levels tested for the analyte in the analyzed sample solution (3.0 $\times$ 10^{-6}; 6.0 $\times$ 10^{-6}; 1.2 $\times$ 10^{-5} and 2.5 $\times$ 10^{-5} mol L^{-1}). A chromatogram showing the CyPh III peak from a standard and from a pharmaceutical sample solution is shown in Figure 7.

A solution of cyclofenil (5 $\times$ 10^{-6} mol L^{-1} prepared in the appropriate solvent system) was exposed to UV radiation during 60 min and introduced into the HPLC, right after irradiation and also every 1 h up to 12 h, at 24 h and 30 h after the solution has been taken out of the photochemical reactor. In between measurements, the derivatized solution was stored under refrigeration (4°C) and in the dark. The results indicated that during 30 h the fluorescence measured from CyPh III were statistically similar (Analysis of variance at 95% confidence limit) with a constant characteristic $t_R$ value. A long-time evaluation was also made by measuring fluorescence from the UV irradiated analyte solution once a day up to 10 days after the derivatization procedure. The results indicated no statistical difference among results obtained during the first three days of the experiment. However, in the 4th day, a signal decreasing occurred resulting in a measured signals of about 91% of the original one (the one measured in the 1st day). Signal decreasing changed signal to about 68% of the original one in the last day of the experiment.

Tests were made to evaluate the stability of cyclofenil present in commercial tablets containing cyclofenil. A fraction of a pulverized pool of tablets were left on an open Petri plate exposed to the ambient light while the control pulverized sample fraction was kept in the dark. In the first day and after 25 days, amounts of the samples were placed in methanol to dissolve cyclofenil and then transferred diluted and mixed with borate buffer (pH 10.5; 10 mmol L^{-1}) to achieve the sample solution composition to be injected into the chromatographic system. It was observed no formation of cyclofenil derivatives within these 25 days of the experiment, which indicated good stability of the drug.

Homogeneity study is performed to check if a specific property presents significant differences at different parts of the analyzed material. If there is no statistical difference, the material can be considered homogeneous in terms of such a property, ruling out any possible error in formulation preparation [13]. Homogeneity studies were made using three different packs of the medicine from local drugstores. From each pack, ten tablets were selected and each one was pulverized and divided in two portions. The cyclofenil content from each of the pulverized tablet was extracted in methanol, diluted and mixed with buffer to get the final composition of methanol/borate buffer (pH 10.5; 10 mmol L^{-1}) 60/40% V/V with a final 3 $\times$ 10^{-5} mol L^{-1} concentration (calculated in terms of the original cyclofenil content indicated in the drug instructions). For each of the packs, the other fraction of the ten pulverized tablets were mixed to form a pool from which an amount was selected to extract its cyclofenil content, then prepared in the proper solvent system to achieve 3 $\times$ 10^{-5} mol L^{-1} (calculated in terms of the original cyclofenil content). These solutions (from the three different pool of tablets and from the 30 individual tablets) were exposed to UV in the reactor (60 min) to compare the relative fluorescence intensity of the CyPh III peak. Analyses of variance (95% confidence limit) were made to compare the signal produced by the ten tablets collected from each of the packs. No significant statistical difference were found either between the tablets or when compared to the signal obtained from the pool of tablets. The result showed a good homogeneity of cyclofenil in all of the packs studied. However, the signals produced by the tablets from one of the packs (and also form the pool of these tablets) were about half of the analytical signal produced by the other two packs (Figure 8). This indicated that one of the medicine packs was substandard.

Eliminating the identified substandard pack, the measurement uncertainty from the other two packs were calculated as $U = k (u_c^2 + u_h^2 + u_s^2)^{1/2}$, where: “$U$” is the expanded uncertainty; “$k$” is a coverage factor ($k = 2$); “$u_c^2$” is the characterization uncertainty; “$u_h^2$” is the uncertainty inherent to homogeneity of the material and “$u_s^2$” is the uncertainty inherent to storage (stability). The $u_c$ value was the standard deviation of ten measurements (each measurement from one different tablet) under the same conditions. The $u_h$ value was obtained from the combined standard deviation of the results obtained from tablets of the individual packs of medicine and the pool of tablets. On the stability study, no peak of CyPf III was observed, thus $u_c$ was insignificant. As the $u_h$ value was 2.3 $\times$ 10^{-6} mol L^{-1} and the $u_h$ value was 4.2 $\times$ 10^{-6} mol L^{-1}, the combined uncertainty of the medicine (solution at 3.0 $\times$ 10^{-5} mol L^{-1}) was estimated was be 4.8 $\times$ 10^{-6} mol L^{-1}, which represent 16% of the concentration in the solution studied.
Conclusion

A HPLC based method for the determination of cyclofenil was developed. Fluorescence detection of a stable cyclofenil photochemical derivative (CyPh III) was made at 328/374 nm leading to an analyte LOD value of $6.7 \times 10^{-8}$ mol L$^{-1}$. The developed method presents a very simple derivatization procedure, avoiding the use of expensive and toxic chemical derivatization agents. The employed chromatographic conditions are very simple. The method allowed the proper quantification of cyclofenil in pharmaceutical formulations and to prove the homogeneity of the drug in commercial formulations. A detailed uncertainty study indicated satisfactory values at $10^{-6}$ and $10^{-5}$ mol L$^{-1}$ range of concentrations provided by the method. This was the first work in literature to provide such a metrological evaluation for the determination of cyclofenil.

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