Automated Chemiluminometric Screening of Counterfeit Drugs of the Antituberculosis Agent Pyrazinamide

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Finding counterfeit drugs presents a growing challenge in preventing these products from entering health systems and causing serious consequences for consumers, drug manufacturers, and governments. In this investigation a simple, low-cost, and expeditious chemiluminometric approach, relying on a fully automated multipumping flow system for screening pharmaceutical preparations of the antituberculosis drug pyrazinamide, was implemented. The developed chemiluminescent method was based on the scavenging effect of pyrazinamide on the oxidation of luminol by hydrogen peroxide in alkaline medium. For analytical signal monitoring, a homemade chemiluminescence detector relying on a photomultiplier module was developed. Linear calibration plots for pyrazinamide concentrations between 10 and 70 mg/L were obtained (R = 0.9931) with good precision (RSD < 0.99%; n = 21). The limit of detection was 5.79 mg/L, and the sampling rate was about 150 determinations per hour.

At this time, the quality of drugs and therefore their effectiveness and safety are less and less certain, especially for the poorest populations, who are attracted by low-priced drugs sold through venues other than pharmacies. Recent years have seen an increase in the prevalence of counterfeit and substandard drugs on the market. The World Health Organization (WHO) has defined counterfeit drugs as those with the correct active ingredients but fake packaging, with insufficient or no active ingredients, or with incorrect ingredients. WHO defines substandard drugs as those produced with little or no attention to good manufacturing practices. Illegal drug rings seem inclined to manufacture either copies with the appearance of known trademarked drugs or substandard or inadequate pharmaceuticals, including generic drugs. Poor quality may be accidental, with no intention to deceive, but oversights in manufacturing or neglected controls—particularly in terms of dosage—can have tragic consequences. This is particularly true in dealing with such a pathogenic and fast-spreading disease as tuberculosis, a major public health problem in developing countries that WHO declared a world emergency in 1993.

Pyrazinamide (PZA) is one of the frontline drugs for treating tuberculosis. The ineffectiveness of monotherapy programs and the outbreak of multidrug-resistant tuberculosis have prompted WHO to recommend 6- or 7-month short-course treatment regimens comprising rifampicin, isoniazid, PZA, and ethambutol. In this context, PZA is an important antituberculosis drug because it helps to shorten the duration of the chemotherapy regimens (1). The therapeutic importance of PZA, along with the hazardous side effects associated with its use—mainly in terms of liver toxicity—and the unpredictable drug half-life that might be prolonged in patients with impaired renal or hepatic function, have prompted the development of distinct analytical methodologies for monitoring this antituberculosis drug. Among these methodologies, the most used are GC (2–4), LC (5–15), potentiometry (16, 17), and UV spectrophotometry, usually associated with chemometric methods (18–21).

An imperative issue demanding simple, low-cost, accurate, and easily automated procedures for PZA determination requires the assessment of pharmaceuticals quality in terms of good manufacturing practices and the search for counterfeit drugs. In effect, many developing countries do not have the technical, financial, or human resources required to apply such standards, leaving them, therefore, more vulnerable to fraud. Meanwhile, some developed countries may be less strict when the product being manufactured is destined for exportation versus domestic use. As a consequence, poor-quality PZA pharmaceuticals, mainly faulty in terms of inadequate dosage, can significantly reduce the effect of the therapeutic regimens, providing no benefit to the patients or even worsening their medical conditions, and thus making it even more difficult to control the spread of the disease.

In the last decades, flow analysis has demonstrated itself to be a continuously evolving field and a valuable tool for implementing expeditious analytical methodologies that provide analytical chemists with alternatives that most of the time are less expensive, simpler, faster, safer, and more easily operated than the conventional procedures because of the higher automation level attained. The automation of analytical
processes for the control of pharmaceutical preparations is an even greater priority, leading to the attainment of increased sample throughputs, high versatility, and the maximization of the equipment’s analytical performance, either in terms of result quality or operation costs.

Multipumping flow systems (MPFS) are a recently proposed flow approach (22) that combines a low cost of implementation, operation, and maintenance; high versatility; and ease of automation with full control of the solution’s manipulation within flow tubing, thus ensuring fast reaction zone implementation, homogenization, and detection. The characteristic pulsed pattern of the flow in MPFS provides an improved homogenization of the solutions inside the flow system, which results from the chaotic movement of solutions during transport. These advantageous features facilitate performing chemiluminescence (CL) measurements, because light is usually generated by fast reactions that require fast and efficient sample–reagent mixing in front of the detector. This way, it is possible to benefit from the sensitivity, selectivity, and equipment simplicity of chemiluminometric methods to implement a novel automated flow methodology for PZA determination, which can be used as a quality assessment tool in detecting counterfeit pharmaceuticals.

**Experimental**

**Reagents**

All chemicals used were of analytical reagent grade, and doubly deionized water was used throughout.

A 50 mg/L stock solution of copper (II) was prepared from CuCl2·2H2O (Fluka, Sintra, Portugal), in a 4 × 10⁻³ M solution of sulfuric acid (Merck).

A 10 × 10⁻³ M luminol (Sigma, Sintra, Portugal) stock solution was prepared in 0.0175 M NaOH (Sigma) and kept protected from light. A 2.5 × 10⁻³ M hydrogen peroxide (Merck, Lisbon, Portugal) solution was prepared on a daily basis.

A 500 mg/L PZA (Sigma) stock solution was prepared by direct dissolution in doubly deionized water. Working standards were prepared daily by appropriate dilution with water.

The validity of the proposed methodology was verified through the determination of PZA content in Pramide® capsules (Irex, Lisbon, Portugal) containing 500 mg of the drug per capsule.

**Equipment**

All measurements were performed by using a homemade continuous flow CL detector (23). These units are essentially characterized by simplicity and low cost compared to other CL detectors such as commercially available ones or those that work by removing the light emission source and exploit only the detection unit (24). For this purpose, an HS5784-04 Hamamatsu (Hertfordshire, UK) photosensor module (PSM) was assembled with a spiral-shaped flow cell (40 μL internal volume) constructed in Perspex®, which was attached to the PSM window and placed inside a light-tight box. The CL detector was powered with ±12 V, and the photomultiplier gain adjustment was performed by means of an external 10 kW potentiometer. The flow manifold comprised 4 fixed displacement diaphragm-type, solenoid actuated micropumps (Ref. 090SP-BIOCHEM, Valve Inc., Boonton, NJ) dispensing 8 μL per stroke. An interface card Advantech PC-LABCard model PCL-818L and a CoolDrive™ power drive board (NRResearch Inc., West Caldwell, NJ) were used to activate the micropumps through electric signals at the Transistor-Transistor Logic pattern. The PCL-818L interface card has a 12-bit analog-to-digital converter that was used for CL analytical signal acquisition and processing.

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**Table 1. Operating parameters of the multipumping flow system**

<table>
<thead>
<tr>
<th>Step</th>
<th>P₁</th>
<th>P₂</th>
<th>P₃</th>
<th>P₄</th>
<th>No. of pulses</th>
<th>Pulse time, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>On</td>
<td>On</td>
<td>Off</td>
<td>Off</td>
<td>10</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>Off</td>
<td>Off</td>
<td>On</td>
<td>On</td>
<td>4</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>On</td>
<td>Off</td>
<td>Off</td>
<td>Off</td>
<td>50</td>
<td>0.3</td>
</tr>
</tbody>
</table>
PTFE, 0.8 mm id, tubing and homemade confluence connectors were also used. Control of the analytical system was accomplished by means of an Intel Pentium-based microcomputer and software developed using MS Quick-Basic Version 4.5.

Flow Manifold and Procedure

The MPFS (Figure 1) comprised 4 self-priming solenoid micropumps (P1 to P4). The micropumps were used to propel each individual solution; they were the only active elements in the flow manifold participating in the CL reaction. When the controllers were activated, the micropumps were filled up (upon aspiration) with a given volume of solution (depending on the micropumping stroke volume). As soon as they were deactivated, the same solution volume was delivered (by propulsion) into the system. Thus, through combining micropumps with appropriate stroke volume with predetermined activation frequencies (controlled in terms of the time interval between successive activations), a wide range of flow rate values could be established. Additionally, because each of the micropumps was controlled independently of the others, the flow rate could be individually programmed for each micropump, which represented an advantage over conventional flow injection analysis systems based on a single peristaltic pump. The simplicity of micropump automation and control allowed the manipulation of the sample and reagent’s volume, flow rate, sampling time, and sampling approach merely through software adjustment.

The analytical system was conceived by taking into account the characteristic modular structure of the MPFS. This way, the manifold, involving the implementation of a 2-stage determination reaction, was designed in 2 substructures: one responsible for luminol and hydrogen peroxide solution mixing and another for the reaction between the sample and Cu(II). The resulting mixed solutions merged into a second reaction zone prior to detection. Micropumps P1 and P2 were the propelling devices for luminol and hydrogen peroxide solutions, which were mixed in reactor L3 and used to establish a baseline. By actuating micropumps P3 and P4, a given volume of both the Cu(II) and sample solutions was inserted in the system, establishing a first reaction zone within L2. The luminol–H2O2 and the Cu(II)–sample solutions merged within L1, originating a second stage reaction and a CL emission that was measured by the CL detector.

The influence of the concentration and composition of the reagents, available time for reaction development, and pH were investigated by injecting triplicate PZA standard solutions. Flow rates, sample–reagent volumes, and mean available time for reaction development were studied by modifying the operating parameters in Table 1.

Reference Method

In order to evaluate the accuracy of the results obtained with the developed procedure, comparative determination of PZA in the same pharmaceutical formulation (Pramide®) was also performed using a standard method, as recommended by the Portuguese Pharmacopoeia (25). This procedure involved a nonaqueous potentiometric titration with 0.1 M perchloric acid.

Results and Discussion

The developed chemiluminescent methodology was based on the oxidation of luminol by hydrogen peroxide, catalyzed by Cu(II). PZA acted as a Cu(II) scavenger by complexing Cu(II) ions, which produced a pronounced inhibition of the CL signal generated by the luminol–hydrogen peroxide reaction. The obtained CL quenching was used to quantify the PZA contents in pharmaceutical formulations.

The automated flow system for PZA determination, which involved the introduction of 4 solutions, was designed and optimized taking into consideration that the CL response was generated by a very fast reaction. Consequently, the sample and reagent’s intermixing would have to be achieved rapidly in order to provide a suitable reaction time prior to detection.
The hydrogen peroxide concentration was varied in the concentration range $1 \times 10^{-3}$ to $10 \times 10^{-3}$ M. It was verified that the maximum CL intensity was obtained at $4 \times 10^{-3}$ M. The concentration of NaOH was a very important parameter because it influenced the pH of the sample zone and therefore the development of the CL reaction. It was verified that from 0.013 to 0.0175 M the analytical signal increased, then subsequently decreased for higher concentrations. Therefore, a 0.0175 M NaOH concentration was selected for the experiments. In close relation to the previous assay, several concentrations of sulfuric acid solutions ranging from $1 \times 10^{-3}$ to $6 \times 10^{-3}$ M were also evaluated. It was observed that the maximum CL signal was attained at $4 \times 10^{-3}$ M, while for lower and higher concentrations of the acid the signal decreased.

The hydrogen peroxide concentration was varied in the range $0.5 \times 10^{-3}$ to $6 \times 10^{-3}$ M, and in that range a continuously increasing CL signal was obtained. Nevertheless, it was observed that for concentration values higher than $2.5 \times 10^{-3}$ M, the signal variation was not as pronounced as for lower concentrations. Thus, an $\text{H}_2\text{O}_2$ concentration of $2.5 \times 10^{-3}$ M was chosen as the working solution.

The Cu(II) mass concentration was evaluated in the range of 1–19 mg/L. A 4 mg/L mass concentration for Cu(II) was chosen, because for higher concentrations the signal tended to stabilize.

**Flow System Parameters**

One approach for maximizing the CL signal was to study the influence of reactors $L_1$, $L_2$, and $L_3$ on the development of the CL reaction. The presence of reactor $L_1$ influences the final stage of the reaction, when copper catalyzes the oxidation of luminol by hydrogen peroxide. Taking into account the high rate of the implemented CL reaction and the improvement of the sample–reactant’s mixing from the micropump’s pulsed flow, it could be expected that it would not be necessary to use any reactor prior to detection. However, other factors can influence its demand, such as the flow rate (hence, time of reaction) and homogenization efficiency, which depends on the flow approach used for mixing the solution Cu(II)–sample with the luminol–$\text{H}_2\text{O}_2$ baseline stream. The study of the influence of $L_1$ reactor length on the CL signal revealed that the 0.15 m length of flow tubing provided the highest analytical signal. For lower and higher values the analytical signal was inferior.

The reactor $L_2$ influenced the homogenization extension between the solutions of Cu(II) and the sample, and thus, the availability of Cu(II) ions for the catalysis of the CL reaction between luminol and hydrogen peroxide. Hence, the optimization of the reactor $L_2$ length was focused on obtaining the minimum CL signal, indicating that the best conditions for the reaction between Cu(II) and the sample were achieved. A length of 0.25 m for the $L_2$ reactor was chosen, taking into account that for higher values the CL signal stabilized.

The presence of reactor $L_3$ in the flow manifold revealed that a maximization of the analytical signal was obtained when a reactor with a 0.25 m length was used. The reactor $L_3$ promoted an improved and efficient mixture between the luminol and hydrogen peroxide solutions, contributing to an enhanced reaction development when all solutions were mixed and the reaction zone transported toward detection.

The following optimization assay globally involves a set of flow parameters consisting of flow rate, sample volume, and sample insertion technique. The influence of the sample insertion technique on the reaction development was studied for different flow rates and for increasing sample volumes. This approach to optimizing the above parameters was possible mainly because the easy control and operation of the flow system by computer allowed for easy manipulation of characteristics directly related to the potential for automation of the multipumping systems (27). The software controlling the system enabled the individual control of the micropumps, and because each one handles each solution involved in the analysis, diverse software configurations for system operation were accessible for use. The physical configuration of the
The manifold described earlier was chosen with the goal to provide easily manipulation of the system and hence, of the solutions involved, taking into account the importance of the prereaction of Cu(II) ions with PZA.

The influence on the CL signal of different approaches for the Cu(II)–sample solution introduction into the baseline flow (luminol–hydrogen peroxide solutions) was studied, involving unique sample volumes, binary sampling, and merging zones. For this purpose, the mixing of Cu(II) and PZA solutions was performed by the simultaneous actuation of micropumps P3 and P4, and the same procedure was applied for the baseline stream of luminol and hydrogen peroxide solutions. The sampling strategy studied was focused on confluence point X1 (Figure 1), where the inhibition by PZA of copper catalysis of luminol oxidation took place.

To study the influence of sampling at confluence point X1 on the overall analytical signal by means of a unique volumes technique, a solution aliquot composed of PZA and Cu(II) was inserted by interrupting the continuous baseline stream with a continuous stream of Cu(II)–PZA solution, so that 2 reaction interface zones were formed. The highest CL signal was observed when 4 pulses of sample were inserted at a pulse time of 0.3 s (Figure 2); an equivalent signal was obtained for 5 pulses of sample, but with 0.5 and 0.7 s of pulse time. These results are consistent with the fact that for lower flow rates (higher pulse times), the time necessary for the reaction zone to reach the detector cell is higher, and so the maximum intensity of emitted light from the reaction occurs outside the flow cell. In this case, the analytical signal is compensated for by increasing the quantity of reagents introduced into the flow system through the insertion of a higher number of pulses during sampling.

To evaluate the merging zones approach, Cu(II) and PZA solutions were merged into the baseline flow of the luminol and hydrogen peroxide solution, and in order to compensate the doubling effect of flow rate during the sampling stage at confluence point X1, the time actuation between pulses of the solenoid micropumps was reduced by a factor of 2. For all the sample volumes, a drop in analytical signal was observed with diminishing flow rates. This means that in this flow sampling situation, there is a very rapid occurrence of reaction immediately upon Cu(II)–sample solution insertion, and any decrease in flow rate would determine higher dispersion effects with negative consequences in the CL signal.

The binary sampling assay was accomplished by alternating the actuation of the micropumps; luminol and hydrogen peroxide solution strokes were alternated with Cu(II) and PZA solution strokes. This assay revealed that, similarly to the merging zones technique, when the flow rate diminishes, the CL signal decreases. When performing the global analysis of the results obtained in the study of the sampling strategy applied to the analytical signal, it was concluded that the insertion of the sample by the unique volumes approach provided the higher CL signal, and that 4 pulses of Cu(II)–PZA solution introduced in the system with a pulse time of 0.3 s supports best the efficiency.

To evaluate the developed methodology for determining PZA in a pharmaceutical formulation (Pramide, the only formulation available), a study of the influence of some compounds normally used as excipients was performed. A compound was considered as noninterfering when the analytical signal variation was ≤3% when compared with the signal obtained in the absence of the referred compound. The results revealed that the presence of the excipients studied—namely cellulose, lactose, starch, and calcium stearate—upon a 100-fold molar ratio regarding PZA did not interfere.

Applications

Before evaluating the developed methodology for determining PZA in a pharmaceutical formulation (Pramide, the only formulation available), a study of the influence of some compounds normally used as excipients was performed. A compound was considered as noninterfering when the analytical signal variation was ≤3% when compared with the signal obtained in the absence of the referred compound. The results revealed that the presence of the excipients studied—namely cellulose, lactose, starch, and calcium stearate—upon a 100-fold molar ratio regarding PZA did not interfere.

Table 2. Operating parameters of the multipumping flow system that make it possible to obtain a linear response range between 1 and 15 mg/L

<table>
<thead>
<tr>
<th>Step</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>Pulse number</th>
<th>Pulse time, s</th>
</tr>
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<td>0.3</td>
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<td>0.3</td>
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<tr>
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</tr>
<tr>
<td>4</td>
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</tr>
<tr>
<td>5</td>
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</tr>
<tr>
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<td>Off</td>
<td>30</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The mechanism for the indirect determination of an organic ligand by means of complexation with a metal ion has been studied previously (28), predicting a sigmoidal shape for the CL intensity as a function of the logarithm of the analyte concentration. The results obtained in this work are in...
agreement with those previous findings, and the plots of CL intensity versus the logarithm of the analyte concentration were sigmoidal.

After system optimization, a linear relationship between CL intensity and analyte concentration was observed for PZA concentrations ranging from 10 to 70 mg/L (Figure 3). This was considered appropriate for performing the analysis of this drug in pharmaceutical preparations, making unnecessary any additional treatment of the sample, namely a preconcentration step. The calibration curve was expressed as $H = -0.2055 \times C + 20.611$, where $H$ represents the peak’s height (in cm) and $C$ is the concentration of the analyte (in mg/L). The correlation coefficient was 0.9931. In order to assess the validity of the proposed methodology, the pharmaceutical formulation Pramide was analyzed through the MPFS, and the obtained results showed a good agreement with those provided by the reference procedure, giving a relative deviation, expressed in percentage, lower than 0.3%, as well as good precision, since it obtained a relative standard deviation of $<0.99\%$ for 21 consecutive determinations. The sampling rate was about 150 determinations/h; we recommend conducting a calibration procedure on the system at the beginning of each workday.

**Exploiting the Multipumping System**

By applying the same MPFS (Figure 1) without performing any physical or chemical modifications on experimental conditions, a calibration curve from 1 to 15 mg/L of PZA (Figure 4), with a correlation coefficient of 0.9965 and with a linear equation represented by $H = -0.5807 \times C + 12.476$ (the variables are the same as above), was achieved through modifying the flow system parameters during the sampling stage. This was done by altering the actuated pulse numbers of the Cu(II) and sample solenoid micropumps, enabling the intercalation of different volumes of the reagent solutions. The ratio of Cu(II) and the sample in the sampling zone that was injected for detection was modified through the software that controlled the system, thus successfully eliminating the need to use other reagent modifications of the manifold system are necessary. Thus, the developed flow methodology can be applied to pharmaceutical samples as well as biological samples. Nevertheless, its application for biological samples is conditioned by other factors that determine the final concentration of PZA, such as the dosage taken and the ratio of metabolization of the drug inside the organism.

**Conclusions**

The developed methodology was revealed to be a valuable strategy for determining PZA in pharmaceutical formulations, and it proved to be a good alternative to other available analysis procedures because it exhibits an elevated degree of automation. It is a precise and simple methodology, it originates a low volume of residues, and it only requires minor intervention by an operator.

Additionally, the coupling of a CL detector to a flow analysis system based on the multipumping concept resulted in a flow methodology with high versatility because it allowed the analyst to work with 2 possible and different linear response ranges for determining PZA. This was enabled through the configuration of different flow control parameters, thus originating variable degrees of sample dispersion. As a result, different concentrations of PZA and reagents can be adjusted during the sampling stage. This was done by altering the operating versatility has the potential to be further explored for application to biological samples, in which the drug quantities assume low-level values. No physical modifications of the manifold system are necessary. Thus, the developed flow methodology can be applied to pharmaceutical samples as well as biological samples. Nevertheless, its application for biological samples is conditioned by other factors that determine the final concentration of PZA, such as the dosage taken and the ratio of metabolization of the drug inside the organism.

**References**


