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Chemiluminometric determination of carvedilol in a multi-pumping flow system

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Abstract

In this work a simple, fast, sensitive and selective flow-based procedure for the chemiluminometric determination of carvedilol, a recent non-cardioselective β-blocker with noteworthy antioxidant activity, is proposed. The developed methodology takes advantage of the antioxidant capacity of carvedilol to inhibit the chemiluminescence response resulting from the oxidation of luminol by hypochlorite, by acting as a hypochlorite scavenger. The analytical process was implemented in a multi-pumping flow system that employs multiple solenoid actuated micro-pumps as the only active components. These acted as solution insertion, propelling and commuting units assuring an easily controlled, low cost, compact and reliable analytical system.

A linear working range for carvedilol concentrations ranging from 1.2×10^{-7} to 3.0×10^{-6} mol l^{-1} ($r > 0.999$, $n = 6$), was obtained, with a detection limit of 8.7×10^{-9} mol l^{-1}. The system handles about 65 samples per hour yielding precise results (R.S.D. < 1.3%, $n = 10$). Recoveries within 95 and 104% were obtained.

Keywords: Multi-pumping flow system; Chemiluminescence; Carvedilol; Luminol

1. Introduction

Carvedilol (±)-1-(carbazol-4-yloxy)-3-[[2-(*O*-methoxyphenoxy)ethyl]amino]-2-propanol, is a non-cardioselective β-blocker that has been recently prescribed for controlling the hypertension and angina pectoris. It exhibits vasodilator properties that are mainly attributed to its blocking activity at the α_1-adrenergic receptors; at higher doses calcium-channel blocking activity may become relevant [1]. Lately, it has been recognised as an effective agent for the treatment of congestive heart failure (CHF). In view of its antioxidant activity [2,3], carvedilol is efficient to suppress lipid peroxidation, protein oxidation or to inhibit the generation of reactive oxygen species [4,5]. It is beneficial for patients with CHF due to its protective effect of the heart [6].

Since its use for pharmaceutical purposes is relatively recent, the methods available for carvedilol determination are scarce. In this context, liquid chromatography and capillary electrophoresis applied to the analysis of biological samples like plasma and serum [7–11] and urine [12] should be highlighted. For analysis of pharmaceuticals, two fluorimetric methods were proposed for the determination of carvedilol [13,14]. Recently, a differential pulse voltammetric procedure using a glassy carbon electrode was developed for the analysis of tablets [15].

The above-mentioned methods require expensive equipment, skilled analysts, laborious sample preparations and/or time-consuming analytical processes being then less suitable for routine analysis. For routine laboratory quality control, for research tasks and/or for rapid screening of biological samples, development of fast, simple, reliable and rugged analytical procedures are required. High quality chemical data collected at run-time is essential for the control of modern chemical manufacturing facilities. Ideally, instru-

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mental techniques should be versatile, capable of accommodating a wide variety of assays without the need for system reconfiguration. On the other hand, a growing tendency towards more compact analytical systems is nowadays manifested. Compactness, considered in terms of less active components performing identical or increased number of tasks, combined with miniaturisation leads to noteworthy advantages, such as enhanced portability, ease of automation and attainment of potentially higher automation levels [16]. Moreover, miniaturised analytical systems are intrinsically more environmentally friendly due to the minimal reagent consumption and thus to the production of lower waste amounts.

Chemiluminescence (CL) has receiving increasingly attention due to the superior detection limits, which are related to distinct aspects like the non-existence of a radiation source and thus the virtual absence of background emission or instabilities of the light source, as well as low signal/noise ratios. It exhibits often wide linear dynamical ranges and makes use of simple instrumentation. CL can be exploited in terms of signal inhibition or enhancement for the determination of a variety of substances. Luminol, a very commonly used compound, can be oxidised in alkaline medium to produce a CL response. Several chemical species were observed to increase or reduce the luminol CL, a feature that has been frequently used with analytical purposes [17–21].

Efficient mixing in close proximity to the detector is crucial for detection systems based on rapid CL reactions, as measurements around the maximum emission yield enhanced sensitivity. In this regard, the fast reproducible sample/reagent mixing provided by multi-pumping flow systems [22], owing to the solenoid micro-pumps generated pulsed flow, is particularly advantageous as it enables the immediate presentation of the reaction zone to the detector ensuring an adequate measurement of the light emitted from the short-lived excited state intermediates produced in the chemiluminescent reaction. Moreover, the correspondent scale down of the analytical system assured both faster flushing and cleaning. These features lead to a significant reduction of the required sample and reagent solutions and to a lessening in the volumes of liquid wastes, ensuring at the same time high sampling rates.

The aim of the work was therefore to develop a multi-pumping flow system for chemiluminometric carvedilol determination with favourable characteristics of robustness, simplicity, selectivity and sample throughput. Lessening of sample and reagent consumption was also aimed.

2. Experimental

2.1. Solutions

All reagents were of analytical grade and deionised water (conductivity <0.1 $\mu S\,cm^{-1}$) was used throughout.

Bulk carvedilol drug was supplied by Roche Farmacêutica Quimica Ltd. (Lisboa, Portugal). A $6.15 \times 10^{-4}\,mol\,l^{-1}$ carvedilol stock solution was prepared by dissolving 6.25 mg of the drug in 1 ml of 4.0% (m/v) ethanol and completing the volume up to 25 ml with water. This stock solution was stored in the refrigerator and protected from light. Working standard solutions with carvedilol concentrations ranging from 1.2×10^{-7} to $3.0 \times 10^{-6}\,mol\,l^{-1}$ were daily prepared by water dilutions of the above stock solution.

The $0.1\,mol\,l^{-1}$ carbonate buffer (pH 10.5) was prepared by dissolving 10.59 g K_2CO_3 in about 800 ml of water, adjusting the pH to 10.5 with $0.1\,mol\,l^{-1}$ HCl, and completing the volume to 1000 ml with water.

The $5.0 \times 10^{-3}\,mol\,l^{-1}$ luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) reagent was prepared by dissolving 88.8 mg of the compound in 100 ml of carbonate buffer (pH 10.5).

The $0.015\,mol\,l^{-1}$ hypochlorite solution (Fluka), was daily prepared. It was standardized according to the Portuguese Pharmacopoeia by titration with thiosulphate [23].

2.2. Apparatus

CL measurements were performed in a Camspec CL-2 chemiluminescence detector (Cambridge, UK) equipped with a three-port quartz flow-cell (60 μl inner volume, 5.0 mm optical path).

The Bio-Chem 090SP solenoid micro-pumps (Boonton, USA) were of the fixed diaphragm displacement type, and presented a stroke volume of 8 μl. An NResearch CoolDriver™ (West Caldwell, USA) power driver was used to operate the pumps.

Control of the analytical system, data acquisition and processing were accomplished through a 486DX-based microcomputer furnished with a PCL-711B Advantech interface card (Taipei, Taiwan). Software was developed in QuickBASIC 4.5.

Transmission lines and reaction coils were made from 0.8 mm i.d. PTFE tubing. End-fittings, connectors and confluence points were also used.

2.3. Flow manifold

The flow system (Fig. 1) was designed with three solenoid micro-pumps (P_1, P_2 and P_3) as the only active devices. Sample and R_1 reagent merged at confluence point 'x' and a second confluent connector was not needed because the flow-cell was a three-port one. As a multi-pumping flow system was concerned, no specific solution insertion valves were required, and this aspect permitted an easier system build-up and control. The solenoid micro-pumps were accountable for both solutions insertion and propelling: P_1, P_2 and P_3 were needed for inserting the sample (S), the hypochlorite reagent (R_1) and the luminol reagent (R_2), respectively. The pulse frequency in combination with the stroke volume determined flow rate, therefore a pulse-counting routine

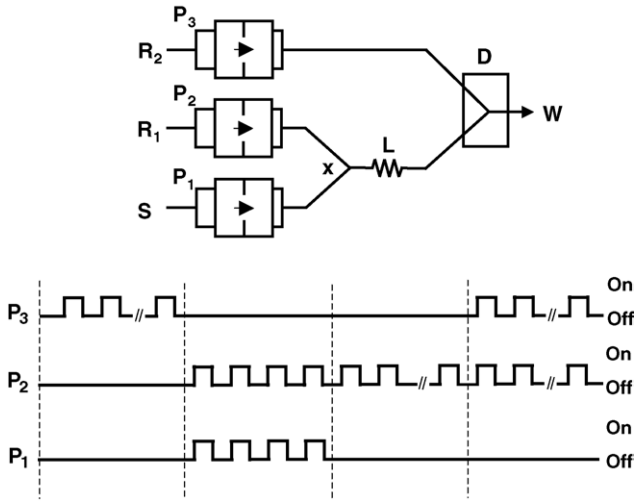

Fig. 1. Flow diagram of the multi-pumping flow system (upper) with indication of the temporal modifications of the pump status (lower). P_1, P_2 and P_3 = solenoid micro-pumps; S = sample; R_1 = hypochlorite reagent; R_2 = luminol reagent; *L* = reaction coil (50 cm); x = confluence point; D = detector; ON = pump filling up; OFF = solution delivery.

defined the volume of each solution inserted in the analytical path.

The analytical cycle (Fig. 1, lower) was started by operating P_3 micro-pump in order to propel the luminol solution through the analytical path, thus establishing baseline. Thereafter, a given sample volume was inserted by actuation of P_1 while the hypochlorite reagent was simultaneously added by means of P_2. Sample and hypochlorite zones merged at confluence 'x' and reaction took place inside the following *L* reactor. P_1 pump was then switched off and R_1 reagent was the only solution to push the sample zone towards detection. The sample zone merged with the luminol flowing stream (propelled by P_3) inside the flow-cell allowing the development of the CL reaction. Sample concentration was evaluated in terms of the decrease of the monitored signal (corresponding to the hypochlorite scavenging effect). In this way, the analytical signal related to the blank solution (no hypochlorite inhibition) corresponded to the maximum CL emission.

3. Results and discussion

Considering that CL refers to the emission of light by molecules participating in highly exergonic chemical reactions, generally oxidation reactions, preliminary investigations were undertaken in order to evaluate the possibility of exploiting the carvedilol antioxidant activity to implement a novel method for its determination. The results confirmed this possibility particularly when hypochlorite was used as the oxidant. Luminol was selected as chemiluminogenic species as it exhibited one of the highest CL quantum yields. As the proposed procedure involved CL inhibition, it was decided to maximise blank readings in order to improve sensitivity.

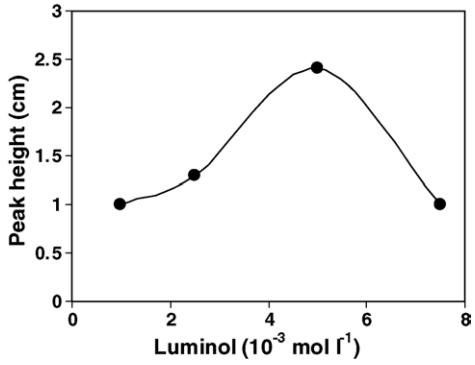

Fig. 2. Influence of luminol concentration.

3.1. Influence of reagents concentration

Effects of luminol and hypochlorite concentrations were investigated by using water instead of the carvedilol solutions. Different concentrations were investigated for both reagents aiming to attain the highest blank values. Luminol concentrations ranged from 1.0×10^{-3} to 7.5×10^{-3} mol l^{-1}, and the results showed that CL emission increased up to 5.0×10^{-3} mol l^{-1} and then decreased (Fig. 2). For this luminol concentration, influence of hypochlorite concentration was investigated within 0.72×10^{-5} and 3.57×10^{-5} mol l^{-1}. The analytical signal underwent a pronounced increase with the hypochlorite concentration and beyond 1.43×10^{-5} mol l^{-1} the detector was overloaded. This value was then selected for further experiments.

As CL reaction is favoured under alkaline conditions, pH was a relevant parameter for system design. Influence of pH was investigated by preparing different luminol solutions in 1.0×10^{-1} mol l^{-1} carbonate buffer with the pH adjusted within 7 and 12. The CL intensity increased with pH up to 10.5 and then markedly decreased (Fig. 3). Taking into consideration this results and following the same principle defined for luminol and hypochlorite concentrations, a pH 10.5 was selected.

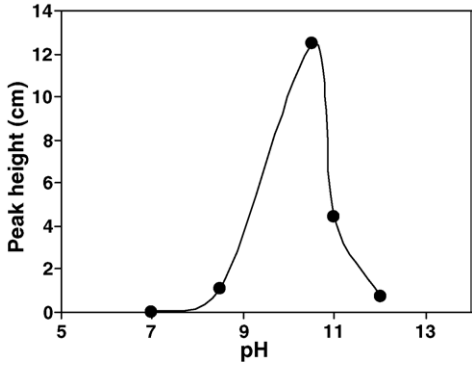

Fig. 3. Influence of pH of the reaction medium.

3.2. Manifold configuration and optimisation

Maximum CL emission occurred immediately after mixing the luminol with the hypochlorite solutions, and light intensity decayed to background level within a few seconds. In order to improve sensitivity, the flow system was then configured (Fig. 1) to assure that the two compounds were put in contact with each other only inside the flow cell. Carvedilol could then be inserted either into the luminol or into the hypochlorite flowing streams. Analysis of the signals obtained with both manifold arrangements revealed that highest inhibition was attained when the sample was previously merged with hypochlorite stream, because it provided a longer available time for reaction development. On the other hand, when the sample was merged with luminol, the inhibition-promoting reaction started only inside the flow cell, and this geometry did not provide enough residence time for reaction development. Moreover, repeatability was poor (R.S.D. > 10%). In this way, the flow system was designed to permit merging of the sample with the hypochlorite stream through the simultaneous actuation of P_1 and P_2 micro-pumps. The number of pulses was maintained for both solutions, therefore sample and R_1 volumes were equivalent, ruled by the number of pulses and the micro-pump stroke volume (8 μl).

When evaluating the influence of sample volume, two complementary objectives were kept in mind: the attainment of high blank readings, which was affected by the number of hypochlorite pulses, and the attainment of a high inhibition that was affected by the number of sample pulses.

By assaying 2–6 sample pulses (corresponding to 16–48 μl) it was observed that the CL inhibition increased with the number of sample pulses. However, above 4 pulses a detector overload impairing detection was noted. The number of pulses was then selected as 4, corresponding to 32 μl.

After defining the sample insertion process, the subsequent task was to synchronise the merging of the first stage carvedilol/hypochlorite zone with the luminol. This synchronisation was important to avoid the continuous addition of luminol, which was only added when the sample zone reached the detector inlet. The pulses for sample transport through *L* coil and detector were successively increased and the highest inhibition was verified when the merging of luminol was carried out after 25 transport pulses. Increasing the number of pulses beyond this value resulted in a significant lessening of the inhibition of the CL intensity.

Flow rate is a relevant parameter especially when the reagents are mixed together inside the flow cell, because it determines the time intervals available for the development of the main reaction and for the detection, which are also dependent on the flow cell geometry. Moreover, influence of flow rate becomes more pronounced for faster CL reactions [24]. In view of the manifold configuration and of the fast luminol-hypochlorite reaction rate, it was noted a pronounced influence of flow rate in the proposed procedure. As a two-stage procedure is involved, influence of flow rate was investigated for both the carvedilol/hypochlorite and the luminol/carvedilol/hypochlorite interactions. In a multipumping system, flow rate is established in terms of pulse frequency that is inversely proportional to period, the time interval between two consecutive pulses. The period was then varied within 0.2 and 1.0 s (300 and 60 s^{-1} frequencies), meaning thus flow rates within 2.4 and 0.48 ml min^{-1}. It was observed that CL intensity increased with flow rate up to 0.8 ml min^{-1} (0.6 s period) and then decreased. It should be noted that variations in the CL intensity were of minor significance in relation to the carvedilol/hypochlorite reaction, but very dependent on flow rate in relation to the luminol/carvedilol/hypochlorite interaction stage (Fig. 4), undoubtedly because it coincided with detection.

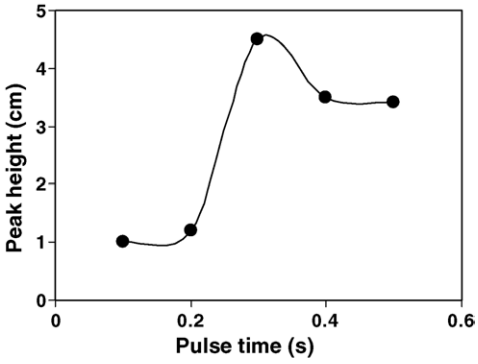

Fig. 4. Influence of the pulse time.

Influence of length of the reaction coil *L*, which determined the mixing conditions and the extent of the reaction of carvedilol with hypochlorite, was investigated up to 100 cm. When only a transmission line was used (*L* = 10 cm) the sample and hypochlorite solutions were immediately mixed with the luminol stream inside the flow cell, and the decrease in the CL intensity was not relevant. As the coil length increased, the CL inhibition was more pronounced reaching a maximum value for a 50 cm coil. The decrease in analytical signal observed for longer coils was due to sample dispersion prior to arrival at the detector.

3.3. Mechanism of inhibition

The mechanism of light generation in the luminol-hypochlorite reaction is intricate. Seemingly, luminol is oxidised by OCl^- to a diazaquinone, which being unstable in aqueous solutions originates the excited aminophthalate [25].

Hypochlorite is a very reactive compound. Carvedilol capacity to inhibit luminol oxidation arises from the fact that the reactivity of ClO^- with luminol is similar to those of amino groups. Accordingly the mechanism of inhibition seems to be related to a competition between carvedilol amino groups and luminol for ClO^-. Since we have favoured carvedilol allowing and increased reaction time between this compound and hypochlorite a significant inhibition effect, even for low carvedilol concentrations was achieved. Inves-

tigations related to a detailed study of the reaction products are beyond the scope of this research, but according to earlier reports related to similar compounds [25] it is probable that hypochlorite reacts with carvedilol amino groups to yield chloramines.

3.4. Analysis of pharmaceutical preparations

After system optimisation, it was applied to the analysis of pharmaceutical preparations with the following operating parameters: 4 sample pulses (32 μl) merging with 4 pulses of 1.43×10^{-5} mol l^{-1} hypochlorite and transported towards detection through a 50 cm reaction coil by using 25 transport pulses before merging with a 5.0×10^{-3} mol l^{-1} luminol solution. Under these conditions, linear analytical curves for carvedilol concentrations within 1.2×10^{-7}–3.0×10^{-6} mol l^{-1} were obtained. A typical curve is represented by:

$$\Delta h = -10.274C - 0.6097$$

where Δh = height of the recorded peak, reflecting the transient variation in the CL intensity; C = carvedilol concentration; $r = 0.9992$; $n = 6$. The detection limit was estimated as 8.7×10^{-9} mol l^{-1} [26]. The relative standard deviation was lower than 1.3% ($n = 10$).

Interferences due to different chemical species commonly used as excipients in pharmaceutical preparations (tablets) were evaluated by using a 1.2×10^{-6} mol l^{-1} carvedilol solution, which was spiked with increasing amounts of the species under evaluation. An excipient was considered as non-interfering if the analytical signal variation was lower than 3% regardless of the CL variation noted in its absence. By assaying up to a 100× excipient/carvedilol molar ratio, it was observed that compounds, such as galactose, lactose, fructose, sucrose, glucose and magnesium stearate did not interfere.

To the best of the authors' knowledge, there is no reference method for carvedilol determination therefore accuracy of results obtained with the proposed procedure was assessed through recovery tests. A given amount of carvedilol was added to solutions of different carvedilol pharmaceutical formulations, and results are shown in Table 1. The recovery data confirmed the absence of interfering matrix effects. Sampling rate was about 200 determinations per hour. Considering that each sample was analysed in triplicate to improve accuracy, this sampling rate corresponded effectively to about 65 samples per hour. Reagent consumption was 0.206 mg of luminol and 0.247 mg of sodium hypochlorite per determination. The system was stable during 8 h working periods and the micro-pumps performance remained unaltered throughout the procedure implementation and accuracy assessment. No baseline drift was observed.

Table 1
Recovery data

Pharmaceutical preparation	Carvedilol (mg l^{-1})		Recovery (%)
	Added	Found	
Dilbloc 25	0.75	0.71	95
RAT 25	0.75	0.78	104
RAT 6.25	0.75	0.74	99
Coronat 25	0.75	0.73	97
Coronat 6.25	0.75	0.72	96

4. Conclusions

The proposed chemiluminometric procedure for the determination of carvedilol in pharmaceutical preparations is characterized by enhanced stability, selectivity, sensitivity, robustness and ease of operation, and these features constitute themselves in a recommendation towards its applicability as an alternative to the scarcely available methods for the determination of this promising β-blocker. In this regard, the developed flow approach exhibits similar or enhanced analytical working range comparatively to the methodologies already available for carvedilol determination in pharmaceuticals.

Implementation of this chemiluminometric reaction in a fully automated multi-pumping flow system resulted in an environment friendly strategy that enabled the reduction of the sample and reagents manipulated volumes as well as of the produced liquid wastes. This leads to a reduction of the costs of the analysis, to an increased repeatability and accuracy, to a lessening of the operator intervention and to a high sampling rate.

Due to the recognised role of carvedilol as antioxidant protector in many oxidative physiological processes the monitoring of carvedilol in biological fluids is presently under investigation.

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