Single reaction interface flow system for chemiluminescent monitoring of mannitol based on its hydroxyl radical scavenger activity

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A B S T R A C T

A single reaction interface flow analysis (SIFA) system for the monitoring of mannitol in pharmaceutical formulations and human urine is presented. The developed approach takes advantage of the mannitol scavenger aptitude to inhibit the chemiluminescent reaction between luminol and myoglobin in the absence of H₂O₂. The SIFA system facilitated the fully automation of the developed methodology, allowing the in-line reproducible handling of chemical species with a very short lifetime as is the case of the hydroxyl radical generated in the abovementioned luminol/myoglobin reaction.

The proposed methodology allowed the determination of mannitol concentrations between 25 mmol L⁻¹ and 1 mol L⁻¹, with good precision (R.S.D. < 4.7%, n = 3) and a sampling frequency of about 60 h⁻¹. The procedure was applied to the determination of mannitol in pharmaceuticals and in human urine samples without any pretreatment process. The results obtained for pharmaceutical formulations were statistically comparable to those provided by the reference method (R.D. < 4.6%); recoveries values obtained in the analysis of spiked urine samples (between 94.9 and 105.3% of the added amount) were also satisfactory.

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1. Introduction

Myoglobin is a small and stable heme protein which contains a single iron protoporphyrin or heme moiety and thus classified as a metalloprotein [1]. It is mainly present in both skeletal and cardiac muscle tissue where it is responsible for oxygen storage and transportation. Several recent works refer that myoglobin in the ferric state Mb(FeIII) could oxidise luminol yielding a chemiluminescence emission. This feature was applied in the determination of several species that could either inhibit or enhance the myoglobin–luminol system [2–4].

It has been also reported that some metal-containing compounds and metalloporphyrins could produce a chemiluminescence response from luminol in alkaline medium, without involving the direct oxidation of the later by myoglobin or the presence of hydrogen peroxide [5]. Seemingly, the reaction between the FeIII porphyrins with NaOH generated hydroxyl radical (OH•) and FeII porphyrins, which could be subsequently oxidised to the FeIII form by O₂ generating superoxide radical (O₂•⁻) [5]. The chemiluminescence emission was therefore a consequence of luminol oxidation induced by the free radicals generated in the redox cycle. Being a metalloporphyrin it would be expected that myoglobin would endure a similar chemical process under the same conditions.

Mannitol is a polyol (sugar alcohol) widely used in food and pharmaceutical industries as well as a therapeutical agent (osmotically active diuretic) used in situations of acute renal failure, to treat cerebral oedema, glaucoma or to reduce intracranial pressure [6]. Mannitol has been determined in different matrices including food, biological samples or pharmaceuticals mainly by chromatography [7–20] and capillary electrophoresis [21–26], using various types of detection, such as, UV, spectrophotometric, electrochemical, amperometric, conductimetric and refractometric. However, the majority of these techniques is time-consuming and requires expensive equipments and qualified operators. Two flow injection analysis (FIA) methodologies involving chemiluminescence detection upon oxidation by permanganate [27] and fluorometric detection [28], were also proposed for the determination of this polyol. However, FIA systems exhibit a low automation level and rely on the continuous addition of reagent solutions to the sample zone, which lead to a substantial consumption of reagents. Furthermore, FIA versatility is limited in terms of sample manipulation since the inserted sample volume is determined by the internal volume of the sample loop.

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Given that mannitol is a specific and strong hydroxyl radical scavenger it would have the ability, by scavenging (OH⋅), to inhibit the anticipated chemiluminescence response generated in the abovementioned myoglobin/luminol reaction, a capability that could be used for its determination in samples with distinct origins. It is relevant the fact that none of the methodologies proposed till now makes use of mannitol radical scavenger capability for its determination.

Chemiluminescence measurements are characterised by wide dynamic linear ranges, high speed of response and excellent sensitivity. Taking into account that a CL response is typically generated by fast reactions, its efficient monitoring requires highly reproducible and fast mixing of sample and reagents. This is even more crucial when short-lived species, as is the case of the hydroxyl radical, are involved in the reactive process. SIFA systems exhibit all the necessary features to fulfil these requisites. Therefore, in this work an automated flow methodology based on a single reaction interface flow analysis (SIFA) system [29] was implemented for monitoring mannitol levels in pharmaceutical and biological samples. The use of solenoid micro-pumps which are accountable for solutions insertion, propelling and commutation, conditioning the establishment and subsequent detection of the reaction zone, provide a great operational simplicity. These micro-pumps are ideal tools to build up compact environment-friendly analytical systems, which are characterised by low consumptions and the minimisation of hazardous waste generation. They also enhance sample/reagent mixing ability in comparison with flow techniques that rely on laminar flow regime. Effectively, solenoid micro-pumps actuation produces a pulsed flowing stream as a consequence of the sudden pump diaphragm displacement that produces a chaotic movement of the solutions in all directions leading to the improved sample/reagent mixing, a well-known requirement of chemiluminescence measurements (fast and effective sample/reagent mixing). Nonetheless, one of the main advantages of SIFA systems is that they no longer rely on the utilisation of well-defined and compelling sample and reagent volumes, a typical characteristic of conventional flow techniques such as FIA, Sequential Injection Analysis (SIA) and Multi-Syringe Flow injection Analysis (MSFIA), but on the establishment of a single sample/reagent reaction interface, where the sample and reagent solutions have no fixed boundaries. This facilitates system configuration and control and resulted in enhanced simplicity and operational versatility while minimises the occurrence of operational errors.

2. Materials and methods

2.1. Reagents and solutions

All of the solutions were prepared with water from a Milli-Q system (conductivity ≤0.1 μS cm⁻¹) and chemicals were of analytical reagent grade quality and not subject to any purification.

A 1.0 × 10⁻³ mol L⁻¹ luminol solution was daily prepared dissolving 8.86 mg in 50 mL of NaOH 0.2 mol L⁻¹. Working standard solutions were prepared by suitable dilutions with NaOH 0.2 mol L⁻¹.

A 1.0 × 10⁻⁵ mol L⁻¹ myoglobin solution was daily prepared dissolving 8.48 mg in 50 mL of water. Working standard solutions were prepared by suitable dilutions with water.

Working standard solutions of mannitol were daily prepared by rigorous dilution of 1 mol L⁻¹ stock solution using water.

Fig. 1. Single interface flow manifold for the determination of mannitol. P₁, P₂, P₃; solenoid micro-pumps; X: confluence point; V₁, V₂: solenoid valves; RC: reaction coils (50 cm); D: chemiluminescence detector; W: waste; Mb: myoglobin (4 × 10⁻⁷ mol L⁻¹); S: sample or standard; L: luminol (1 × 10⁻⁵ mol L⁻¹); prepared in NaOH 0.2 mol L⁻¹.

2.2. Apparatus

The single interface flow system comprised three solenoid micro-pumps (120SP1210-4TE, Bio-Chem Valve Inc., Boonton, NJ, USA, 10 μL per stroke), two 161 T 031 (NRseach, West Caldwell, USA) two-way solenoid valves and a Camspec CL-2 chemiluminescence detector (Camspec Ltd., Cambridge, UK) equipped with a three-port 60 μL inner volume quartz flow cell. The luminometer had a wavelength response range of 320–600 nm and a flow cell working pathlength of 5 mm. Flow lines and reaction coils were made from 0.8 mm i.d. PTFE tubing.

A Pentium-I-based computer was used for system control, and for data acquisition and treatment; software was developed in Microsoft Quick-Basic 4.5. The computer was equipped with a PC-LABCard model PCL-711B interface card from Advantech (Taipei, Taiwan), A CoolDrive (NRseach Inc., West Caldwell, USA) power drive was used to operate both the solenoid micro-pumps and solenoid valves.

Spectrophotometric measurements were carried out in a UV/Vis Spectrometer model Lambda 45 from PerkinElmer Instruments Inc. (Norwalk, CT, USA).

2.3. Single interface flow manifold

The developed flow manifold, pictured in Fig. 1, comprised three solenoid micro-pumps (P₁, P₂ and P₃), which were used to insert and propel the sample and reagent solutions. The repetitive micro-pump switching on/off created a pulsed flowing stream in which the pulse volume corresponded to the micro-pump stroke volume. Two way-(normally closed) solenoid valves (V₁ and V₂) were used to direct the flowing streams. The detector was placed at the centre of the flow manifold. The reactions coils, identically sized, were placed on both sides of the detector.

The analytical cycle was started by establishing a baseline, which was accomplished with myoglobin/sample solutions. For establishing the baseline, V₁ was open and P₁ and P₂ were repeatedly actuated (on/off switching) propelling the solutions through the reactors as well as through the detector. After reaching V₂ these solutions were discarded. Subsequently, V₂ was opened, P₁ and P₂ were switched off and the luminol solution was inserted into the analytical path by means of P₃ and was propelled back to V₁. The mutual interdispersion of myoglobin/sample/luminol resulted in an analytical signal, which was measured when the reaction interface passed through the detector.

2.4. Reference method

Aiming at the evaluation of the accuracy of the results obtained with the developed procedure, mannitol pharmaceutical formulations were analysed according to the British Pharmacopoeia [30], by iodimetric titration.
3. Results and discussion

Preliminary experiments showed that at pH<8 myoglobin exhibits a maximum of absorbance at 409 nm, corresponding to Mb(FeIII) form (metmyoglobin), while at higher pH values the peak maximum was at 414 nm for Mb(FeIII) (oxymyoglobin). It was also observed that the ferric state myoglobin reacted with luminol, in alkaline medium, producing a strong chemiluminescence (CL) emission. This CL emission was markedly reduced when mannitol, a strong and specific hydroxyl radical scavenger, was added confirming that this free radical was involved in the production of light. These results agreed with previous results obtained with other iron porphyrins [5].

3.1. Development of single interface flow methodology

In the development of the single interface flow methodology several studies were carried out in order to improve systems performance, namely in terms of analytical signal intensity, accuracy, repeatability and determination rate. These features influenced the choices made during the optimisation of the systems, which was carried out using the univariate method. Since no well-defined sample or reagent volumes were used, these parameters were not subject to evaluation which simplified the overall optimisation process.

3.1.1. Chemical parameters

Considering that the pH of the solution determined the Fe oxidation state, in order to guarantee the Mb(FeIII) form myoglobin solutions were prepared directly in deionised water at pH 5.6. Effectively, when myoglobin was prepared in alkaline medium a weak CL intensity was observed. On the other hand, luminol CL emission occurred in alkaline conditions. Under these circumstances the SIFA system would have to provide good mixing conditions at the myoglobin/luminol single interface to ensure an appropriate reaction development.

The influence of myoglobin concentration in the chemiluminescence intensity was assayed for concentrations ranging from $1.0 \times 10^{-7}$ to $6.0 \times 10^{-7}$ mol L$^{-1}$. It was observed that with increasing myoglobin concentrations the intensity of the signal increased about 61.5% until myoglobin $4.0 \times 10^{-7}$ mol L$^{-1}$, above which the chemiluminescent signal approached stabilisation. Therefore, a $4.0 \times 10^{-7}$ mol L$^{-1}$ myoglobin solution was used in the subsequent experiments.

The chemiluminescence intensity was also studied for luminol concentrations between $0.1 \times 10^{-5}$ and $5 \times 10^{-5}$ mol L$^{-1}$. The obtained results showed that with increasing luminol concentrations the intensity of the chemiluminescent response increased about 59.1% until $1.0 \times 10^{-5}$ mol L$^{-1}$. Above this concentration value the chemiluminescent intensity remained almost unaffected. Consequently, a $1.0 \times 10^{-5}$ mol L$^{-1}$ luminol solution was chosen for the succeeding studies.

Considering that the luminol oxidation is favoured in alkaline medium, distinct NaOH solutions with increasing concentrations were evaluated. It was observed that the CL emission reached a maximum value for 0.2 mol L$^{-1}$ NaOH.

3.1.2. Physical parameters

By comparing chemiluminescence intensities using reaction coils of different lengths (10–100 cm) in the presence of $1.0 \times 10^{-5}$ mol L$^{-1}$ luminol and $1.0 \times 10^{-7}$ mol L$^{-1}$ myoglobin, it was observed that the chemiluminescence intensity did not diverge significantly. Nevertheless, aiming at guaranteeing a good mixture and in order to not compromise the determination rate two 50-cm reaction coils were selected for the subsequent experiments. Flow rate was evaluated between 0.6 and 3.0 ml min$^{-1}$. Since no significant differences were verified in the chemiluminescence response, a flow rate of 2.0 ml min$^{-1}$ was chosen. These results showed that reaction coil length and flow rate were not fundamental parameters affecting the intensity of the analytical signal obtained with the SIFA system, which could be probably explained by the fact that since sample and reagent solutions have no fixed physical limits any increase in the residence time would be compensated by the continuous surplus of solutions participating in the single reaction interface as it expands to the neighbouring intact (non-dispersed) zones.

3.2. Interferences

In order to assess the selectivity of the developed approach it was applied in the analysis of a 50 mmol L$^{-1}$ mannitol solution containing increasing amounts of several species that are either used as excipients in pharmaceutical formulations or appeared in the composition of human urine samples. A chemical specie was considered as non-interfering if the analytical signal variation was lower than 5% regarding the one obtained in its absence. The obtained results (Table 1) showed no significant interfering effect for the majority of the tested compounds with a tolerable molar concentration ratio of about 100, except for iron(III) which interferes at concentration values similar to those of mannitol. These results confirmed that the proposed methodology can be applied in the analysis of mannitol in both pharmaceutical formulations and urine.

### Table 1

<table>
<thead>
<tr>
<th>Added species</th>
<th>Tolerance limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl$^-$, NO$_3^-$, Ac$^-$, I$^-$, SO$_4^{2-}$, PO$_4^{3-}$</td>
<td>100%</td>
</tr>
<tr>
<td>Na$^+$, Ni$^{2+}$, Zn$^{2+}$, Cr$^{3+}$, Mg$^{2+}$, Ca$^{2+}$, Cu$^{2+}$</td>
<td>100%</td>
</tr>
<tr>
<td>Sorbitol, glucose, borate, oxalate, malate, urea</td>
<td>100%</td>
</tr>
<tr>
<td>Uric acid, NH$_4^+$, BrO$_3^-$</td>
<td>10%</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>1%</td>
</tr>
</tbody>
</table>

Data refer to the concentration ratio (expressed in mol L$^{-1}$) between the interfering specie and the analyte.

### Table 2

Parameters evaluated during the optimisation of the SIFA system performance and most favourable values selected for its operation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Evaluated range</th>
<th>Selected values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactor length (cm)</td>
<td>10–100</td>
<td>50</td>
</tr>
<tr>
<td>Flow rate (ml min$^{-1}$)</td>
<td>0.6–3</td>
<td>2.0</td>
</tr>
<tr>
<td>Myoglobin concentration ($\times 10^{-5}$ mol L$^{-1}$)</td>
<td>1.0–6.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Luminol concentration ($\times 10^{-3}$ mol L$^{-1}$)</td>
<td>0.1–5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Mannitol concentration (mol L$^{-1}$)</td>
<td>$1 \times 10^{-3}$–1</td>
<td>$2.5 \times 10^{-3}$–1</td>
</tr>
</tbody>
</table>

3.3. Application to pharmaceutical formulations and biological samples

After system optimisation and by using the analytical conditions exhibited in Table 2, a linear working range for mannitol concentrations between 25 mmol L$^{-1}$ and 1 mol L$^{-1}$, was obtained (Fig. 2). The calibration curve was expressed by the equation:

$$I = -15.952C + 92.224$$

where $I$ represents the relative chemiluminescence intensity and $C$ is the logarithm of mannitol concentration (expressed in mol L$^{-1}$). The correlation coefficient was 0.9997.

In order to evaluate the applicability and the accuracy of the developed methodology in the analysis of real samples, it was
applied to the determination of mannitol in commercially available pharmaceutical formulations. The obtained results (Table 3) exhibited a good agreement between the results furnished by both methods, with relative deviations (expressed in percentage) lower than 4.6%. Furthermore, the repeatability was good, with a relative standard deviation lower than 4.7% (n = 3), and the determination rate was about 60 h−1. For comparison purposes a paired t-test was also performed on the data obtained by the proposed method and by the reference method. A t value of 0.39 was obtained, which was lower than the tabulated t value = 4.30 (P = 0.05, d.f = 2) indicating no significant differences for the mean concentrations obtained by both methods. When applied in the analysis of human urine samples the developed chemiluminometric SIFA methodology showed as well a good performance, with recovery values (expressed in percentage of the added amount) ranging from 94.9 to 105.3% (Table 4), indicating that the proposed analytical approach is suitable for the monitoring of mannitol in these biological samples.

The developed flow systems exhibited good stability (no baseline drift was observed) and robustness. The utilisation of solenoid micro-pumps guarantees a good operational simplicity, and enhanced sample/reagent mixing ability, an important feature for carrying out chemiluminescence measurements that assure as well low solutions consumption and the minimisation of hazardous waste generation. When compared with more conventional CL flow methodologies SIFA systems revealed a simpler configuration and control while the occurrence of operational errors is minimised because they do not require the reproducible insertion of well-defined sample and reagent volumes.

4. Conclusion

The proposed single interface system allowed fast and reliable determination of mannitol in pharmaceutical formulations and in urine. For moreover, the proposed method has an innovator characteristic since, contrary to the existing methods, it uses mannitol radical scavenger property for its determination, which improves selectivity. The developed system presented an acceptable relative standard deviation that demonstrates a good repeatability. This feature is certainly a consequence of the particular characteristic of SIFA systems that is the establishment of the reaction zone without using definite sample and reagent volumes. When compared to the reference procedure, relative deviations were inferior to 4.6%. The accuracy of the proposed methods face to the reference method was confirmed by paired t-test for pharmaceutical formulations, as well as, the obtained recoveries in urine spiked samples.

The proposed method requires no sample pretreatment, provides a wide working concentration range and is more versatile than most of the reported methods. Furthermore, single interface flow system presents a simpler optimisation than other flow techniques.

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