A continuous flow methodology for the chemiluminometric determination of clomipramine in pharmaceutical preparations is described. Clomipramine acts as a sensitizer on the chemiluminescent oxidation of sulphite by Ce(IV). The developed procedure is based on the multicommutated flow concept whose versatility was fully exploited by using a single solution propelling device for the insertion of multiple solutions in propulsion mode. The utilisation of a binary sampling approach enabled sample/reagent mixing within the flow cell with a significant reagent saving. A time-based sample insertion assured an effective control of sample dispersion, enabling the attainment of distinct working concentration ranges by means of the selection of appropriate sampling times. Linear calibration plots were obtained for clomipramine hydrochloride concentrations ranging 2.5–60 mg l$^{-1}$, depending on sampling time, with good reproducibility and R.S.D. lower than 4.6% ($n = 4$). The developed methodology was applied in the analysis of pharmaceutical preparations and the obtained results were in good agreement with those furnished by the reference procedure with R.D. lower than 3.3% and a sampling rate between 19 and 32 samples per hour.

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Keywords: Multicommutation; Chemiluminescence; Clomipramine; Cerium(IV); Sulphite

1. Introduction

Clomipramine hydrochloride, 3-chloro-5-[3-(dimethylamino)propyl]-10,11-dihydro-5H-dibenz[b,f]azepine monohydrochloride is a dibenzazepine tricyclic antidepressant. Like other tricyclics, clomipramine inhibits noradrenaline and serotonin reuptake in CNS exhibiting antimuscarinic properties as well. Clomipramine is widely used in the treatment of depression, obsessive-compulsive disorders, phobias, anxiety disorders and narcoleptic syndrome [1]. Due to its therapeutical relevance, several methods have been reported for clomipramine determination including spectrophotometry [2–5], thermometric titrimetry [6] and electrochemical method [7]. However, most of the available procedures are based on chromatographic methods [8–10].

Chemiluminometric methods have been subjected to a growing interest since they require simple and low-cost-measuring devices providing a high versatility in the determination of a wide variety of species along with highly sensitive and wide working concentration ranges. Since no external light source is required, the absence of strong background light levels, such as those found in spectrophotometry and fluorimetry, reduces noise signals and leads to improved detection limits. To our knowledge in this specific field, only a liquid chromatography method with post-column tris(2,2′-bipyridyl)ruthenium(III) chemiluminescence (CL) was proposed for clomipramine determination [11].

The oxidation of sulphite by Ce(IV) in sulphuric acid medium is a well-known chemiluminescence reaction [12,13]. We have found that clomipramine acts as a sensitizer for this reaction by increasing the emission intensity. Analytical measurements by CL are very sensitive to multiple experimental factors, and even slight variations of these factors affect the emitted radiation extensively. Moreover, CL is normally produced by fast reactions required for a proper monitoring that the sample/reagent mixing and the succeeding chemical reaction to take place in front of the...
light detector. Accordingly, highly reproducible and fast mixing of sample and reagents is required, a situation usually accomplished by using flow injection analysis (FIA) systems relying on the uninterrupted addition of reagent solutions to the sample zone, which leads to a substantial consumption of very expensive reagents frequently.

The combination of multicommutation with CL gives CL analysis the reproducibility, versatility and degree of automation it requires. Multicommutation allows the implementation of the binary sampling concept, which consists of sampling small aliquots of sample and reagent and inserting them alternately into the analytical path. Dispersion occurs from the multiple liquid interfaces of the aliquots in the reaction coil, allowing a fast intermixing between sample and reagent solutions and improving the reaction development [14,15]. A noteworthy advantage of the developed multicommutated flow systems is that the reagents' addition was precisely focused on the sample zone, thus, avoiding superfluous reagent consumption.

The aim of this work was the development of a multicommutated flow system for the chemiluminometric determination of clomipramine based on the sulphite/Ce(IV) reaction and its application to the analysis of pharmaceutical products.

2. Experimental

2.1. Reagents

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

A 500 mg l\(^{-1}\) clomipramine standard solution was weekly prepared by dissolving 25 mg of clomipramine in 50 ml of deionised water. This solution was kept in the refrigerator. Working standards were daily prepared by diluting 25 mg of clomipramine in 50 ml of deionised water. This solution was kept in the refrigerator. A 1 × 10\(^{-3}\) mol l\(^{-1}\) cerium(IV) solution was prepared by dissolving the required amount of Ce(SO\(_4\))\(_2\)·4H\(_2\)O in 250 ml of H\(_2\)SO\(_4\) 0.15 mol l\(^{-1}\). A 1 × 10\(^{-3}\) mol l\(^{-1}\) sulphite solution was daily prepared by dissolving the required amount of sodium sulphite in 250 ml of deionised water. Pharmaceutical preparations of clomipramine hydrochloride analysed, Anafranil 10, 25 and 75 mg and Anafranil injectable 25 mg/2 ml, were commercially available. Sample solutions were prepared either by dissolving the required amounts of powdered tablets or the required volume of injectable solution in 100 ml of deionised water.

2.2. Apparatus

For chemiluminescence measurements, a Camspec CL-2 (UK) chemiluminescence detector equipped with a 60-\(\mu\)l inner volume flow cell was used. The flow manifold consisted of a set of three-way solenoid valves (161 T031, Research), flow lines and holding coils made of 0.8 mm i.d. PTFE tubing. A Crison Micro BU 2031 automatic burette equipped with a 10-ml syringe and was controlled by a microcomputer through serial protocol (RS-232C) was used to aspirate and to propel the solutions.

A home made power driver based on a ULN 2003 integrated circuit was used to operate the solenoid valves. Data acquisition and control of the analytical system were accomplished through a PC-LABCard model PCL-711B interface card from Advantech and a 486 DX-based microcomputer. The software was developed in QuickBASIC 4.5 and permitted to control the automatic burette and sulphite solvents and data acquisition and processing.

2.3. Flow manifold

The flow network comprised four three-way solenoid valves and is pictured in Fig. 1.

Valve \(V_1\) was responsible for insertion of the sample and the Ce(IV) reagent solution. \(V_2\) was responsible for sulphite insertion, while \(V_1\) and \(V_2\) were used to direct the flow between the two distinct pathways. Initially, the solenoid valves were in position 1 and the Ce(IV) solution that filled the syringe pump, which was also used as carrier solution, was propelled through L\(_1\) towards the detector establishing the baseline. Valve \(V_2\) was actuated to position 2 and the sample was inserted in the analytical path by aspiration into the holding coil L\(_1\). During sample insertion and by operating alternately \(V_4\) between positions 1 and 2, while \(V_2\) was in position 2, very small sample aliquots, were intercalated with very small Ce(IV) aliquots, which created multiple reaction Fig. 1. Flow manifold diagram: \(V_1\), \(V_2\), \(V_3\) and \(V_4\); three-way solenoid valves; solid lines within the valves correspond to position 1 and dashed lines to position 2; P: burette; D: detector; L\(_1\), L\(_2\) and L\(_3\): holding coils; L\(_4\): connecting tubing; W: waste; S: sample; CS: Ce(IV) carrier solution; R\(_1\): Ce(IV) solution; and R\(_2\): sulphite solution.
interfaces and facilitated sample/Ce(IV) mixing. This way L3 was partially filled with a string of sample slugs in tandem with slugs of Ce(IV) solution. The number and timing of each intercalation cycle defined the final sampling time that depending on the flow rate established the sampling volume.

V2 was subsequently moved to position 2 (V2 was moved to position 1) and sulphite solution was aspirated in the holding coil L2, during a pre-defined reagent time interval. Following sulphite insertion, V1 was actuated to position 2 and the solutions within L1 and L2 were alternately propelled towards detection as V1 was repeatedly actuated between position 1 and 2 (the actuation of V1 between the two positions was synchronised with the similar combined actuation of V2 and V3) during pre-set intercalation time intervals. This allowed the intercalation of very small sulphite and clomipramine/Ce(IV) aliquots inside the detector’s cell that resulted in the chemiluminometric reaction development. After sample detection, V1 was actuated to position 1 for clean up and sample replacement.

This analytical procedure was also used to perform the calibration which involved the insertion of a set of standard solutions.

3. Results and discussion

The reaction of Ce(IV) and sulphite produces a very weak CL emission that is attributed to excited sulphur dioxide molecules [17]. However, preliminary studies showed that clomipramine could act as a sensitizer in this reaction increasing CL intensity. A crucial factor in terms of CL emission was the sequence followed for the mixing of clomipramine and reagents solutions. By using the direct reaction between sulphite and Ce(IV) to establish baseline, it was verified that when clomipramine was first mixed with Ce(IV) and subsequently with sulphite, a positive peak was obtained revealing an increase in the CL emission. On the other hand, when clomipramine was first mixed with sulphite followed by Ce(IV), there was a pronounced decrease on CL intensity resulting in a negative peak underneath baseline. Thus, for the subsequent work, the flow manifold had to be designed to guarantee an efficient clomipramine/Ce(IV) mixing prior to the introduction of the sulphite solution.

The implementation of a CL reaction in a continuous flow methodology has two major and complementary requisites: the sample plug has to be efficiently mixed with the reagents and the emitting compound should be produced near the detector cell because usually the reaction rate is very high. When using a FIA-based methodology, the addition of reagents is not subjected to any synchronisation or automated control, and thus, they are usually continuously added, which represents substantial reagent consumption.

A multicommutated flow system exhibits a higher degree of automation with all parameters under computer control, which permits to manipulate reaction zone formation, including added volumes and sequence of addition, thus, reaction development.

When designing a multicommutated flow manifold for the chemiluminometric determination of clomipramine, in order to fulfill the above mentioned requisites, several factors had to be taken into consideration. These factors included not only the chemical parameters but also sample and reagent insertion times, reaction time between clomipramine and Ce(IV), clomipramine-Ce(IV)/sulphite volume ratio and flow rate.

3.1. Sampling time, reagent time and flow rate

An issue of great importance in any flow procedure is sample insertion. Initially the flow network comprised only three solenoid valves: clomipramine was inserted through V3 into L3 by aspiration as a single volume, which was established under a time-based control in terms of a sampling time t1; subsequently the sample zone was propelled by the Ce(IV)/carrier/reagent solution towards detection where the sulphite solution was added. It was observed that, with this modus operandi, each sample insertion originated two distinct peaks because the utilisation of a single sample volume creates only two reaction interfaces and the dispersion within L3 was not enough to guarantee proper sample/Ce(IV) mixing. Immediate alternative would be to increase the length of L3 or to reduce sample volume but the obtained analytical signals demonstrated that these solutions resulted either in an increase of the time required for the analysis, affecting the sampling rate, or in increment of sample dispersion that restrained sensitivity. To overcome this drawback, an extra valve V4 was added, which enabled the insertion of the same sample volume as multiple of very small aliquots that were intercalated with very small aliquots of Ce(IV). The combined intercalation time of each sample aliquot defined the overall sampling time. This way multiple reaction interfaces were created, that facilitated reaction zone homogenisation and permitted to reduce the length of L3. In effect L3 was downsized to the minimum length allowing a physical connection between V3 and the detector. Moreover, as the small sample and Ce(IV) aliquots almost immediately coalesced during sample insertion, the first-stage reaction started without delay enhancing reaction development.

The second-phase reaction, consisting the addition of sulphite to the sample/Ce(IV) reaction zone, was also accomplished at the detector inlet by intercalating very small aliquots of the sulphite solution (defined in terms of
increased as well with its sulphite 1 sequent work 10 cycles of 2-s clomipramine-Ce(IV)/5-s sulphite produced the highest analytical signals. However, for sub-
time, it was possible to manipulate sample disper-
emission increased until a 3 s and then stabilised. With a 50 mg l
of clomipramine solution, the analytical signal increased as well with t5 s, then stabilised between 5 and 8 s and then slightly decreased due to the appearance of two separated peaks resulting from poor reaction zone homogenisation. These results showed that controlling the sampling time, it was possible to manipulate sample disper-
the two solutions participating in the intercalation process, were also subjected to assessment in order to define the most favourable conditions. The evaluation of the influence of clomipramine/Ce(IV) and sulphite/clomipramine–Ce(IV) intercalation strings on reaction development was carried out by assaying different combinations of intercalation cycles/intercalation time in such a way as to keep con-
stant the overall sample time. For clomipramine/Ce(IV) intercalation, the following combinations were assayed: 10 cycles of 1-s clomipramine/1-s Ce(IV), 10 cycles of 1-s clomipramine/2-s Ce(IV), 5 cycles of 2-s clomipramine/1-s Ce(IV) and 5 cycles of 2-s clomipramine/2-s Ce(IV). For sulphite/clomipramine-Ce(IV) string, the intercalation time intervals used were: 20 cycles of 1-s clomipramine-Ce(IV)/5-s sulphite 1 × 10−3 mol l−1 and 10 cycles of 2-s clomipramine-Ce(IV)/5-s sulphite 1 × 10−3 mol l−1. Concerning sample insertion, maximum CL emission was obtained for 10 cycles of 1-s clomipramine/1-s Ce(IV), confirming that small aliquots favoured homogenisation. For sulphite introduction, we verified that 20 cycles of 1-s clomipramine-Ce(IV)/5-s sulphite 1 × 10−3 mol l−1 produced the highest analytical signals. However, for sub-
sequent work 10 cycles of 2-s clomipramine-Ce(IV)/5-s sulphite 1 × 10−3 mol l−1 was selected, as the slightly de-
crease in CL emission was compensated by faster detection and higher sample throughput.

In a chemiluminometric flow methodology, the influence of flow rate is critical as too low or to high flow rates, de-

growing sensitivity, it was selected for the subsequent assays.

The length of the holding coils L1 and L2 had no influence on the analytical signals and these were merely dimen-
sioned long enough as to prevent solutions from reaching the syringe pump.

3.2. Ce(IV) and sulphite concentration

The effect of Ce(IV) concentration on the CL emission was investigated employing a clomipramine standard con-
centration of 50 mg l−1. It was verified that the CL emission increased until 1 × 10−3 mol l−1 Ce(IV) and then sharply decreased, as shown in Fig. 2. According to some authors, the CL emission decreases with higher Ce(IV) concen-
tations because the reaction rate increases and maximum emission occurs before the solution is introduced into the detector flow cell [18,19]. In our case, this does not seem to be a likely justification as the second-phase reaction zone is formed only within the detector. A more plausible expla-
nation is that at high concentrations, the excess of Ce(IV) might absorb a significant amount of the emitted light [20]. Therefore, a 1 × 10−3 mol l−1 Ce(IV) concentration was used for subsequent work.

The influence of sulphite concentration on the emission intensity was studied in the 5 × 10−4 to 0.1 mol l−1 range. It was observed that the analytical signal increased up to a sulphite concentration of 1 × 10−3 mol l−1 and then approached stabilisation.

The CL reaction between Ce(IV) and sulphite occurs in sulphuric acid medium; thus, an important parameter to be optimized was the H2SO4 concentration. The effect of sulphuric acid was evaluated at concentrations up to 1.5 mol l−1 and the results are shown in Fig. 3. Maximum emission in-
tensity was obtained with a 0.15 mol l−1 sulphuric acid so-
3.3. Interferences

Considering that the developed methodology would be applied to the determination of clomipramine in pharmaceutical preparations, the interference effect of several compounds commonly used as excipients was assessed. Samples containing clomipramine at a fixed concentration of 5 mg l\(^{-1}\) and increasing concentration of the excipient were analysed by the developed methodology.

A compound was considered as non-interfering if the analytical signal variation was ±3% when compared to the analytical signal obtained in the absence of the referred compound. The obtained results (Table 1) showed that under the used reaction parameters and at concentrations usually found in the pharmaceutical formulations the studied excipients did not interfere.

### Table 1

<table>
<thead>
<tr>
<th>Interference</th>
<th>Tolerance molar ratio (1.42 \times 10^{-5}) mol l(^{-1}) clomipramine added.</th>
<th>(100^a)</th>
<th>(50^a)</th>
<th>(10^a)</th>
<th>(50^a)</th>
<th>(100^a)</th>
<th>(100^b)</th>
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<tr>
<td>Stearic acid</td>
<td>100</td>
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<tr>
<td>Sucrose</td>
<td>50</td>
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<td>Glycerin</td>
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<td>Starch</td>
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</tr>
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</table>

\(^a\) Highest value tested.

3.4. Analysis of pharmaceutical preparations

After system optimization (Table 2) and by using sampling times of 5 and 10 s, two linear working ranges 30–60 and 2.5–10 mg l\(^{-1}\), respectively, were obtained. For a sampling time of 5 s, the analytical curve was represented as \(A = 0.4909C + 0.3795\) where \(A\) was the peak height expressed in cm and \(C\) was the concentration of clomipramine expressed in mg l\(^{-1}\), with a correlation coefficient of 0.9974, and the R.S.D. was 4.6% (\(n = 4\)). For a 10-s sampling time, the analytical curve was represented as \(A = 1.432C - 1.575\) and the correlation coefficient was 0.9966, and the R.S.D. was 3.7% (\(n = 4\)). The detection limits were 0.65 and 0.7 mg l\(^{-1}\) for the 5- and 10-s sampling times, respectively. Comparatively to a previously developed spectrophotometric flow method [5], the proposed chemiluminometric method is more sensitive and exhibits a higher sampling rate. Moreover, it is more environment friendly requiring lower solutions consumption.

### Table 2

<table>
<thead>
<tr>
<th>Operational parameter</th>
<th>Optimized value</th>
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</thead>
<tbody>
<tr>
<td>Sampling sequence</td>
<td>10 cycles (1 s clomipramine/1 s Ce(IV))</td>
</tr>
<tr>
<td>Reagent sequence</td>
<td>10 cycles (2 s clomipramine–Ce(IV)/5 s sulphite)</td>
</tr>
<tr>
<td>Cerium(IV) concentration (mol l(^{-1}))</td>
<td>(1 \times 10^{-3})</td>
</tr>
<tr>
<td>Sulphite concentration (mol l(^{-1}))</td>
<td>(1 \times 10^{-3})</td>
</tr>
<tr>
<td>Sulphuric acid concentration (mol l(^{-1}))</td>
<td>0.15</td>
</tr>
<tr>
<td>Flow rate (ml min(^{-1}))</td>
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</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration declared (mg/formulation)</th>
<th>Sampling time (s)</th>
<th>Concentration found (mg/formulation)</th>
<th>R.D.(^a) (%)</th>
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<tbody>
<tr>
<td></td>
<td>Developed methodology</td>
<td>Reference method</td>
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<tr>
<td>Anafranil 25 (injectable)</td>
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<td>5</td>
<td>25.27 ± 0.63</td>
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<td></td>
<td></td>
<td>10</td>
<td>24.95 ± 0.92</td>
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<tr>
<td>Anafranil 10 (tablets)</td>
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<td>5</td>
<td>8.96 ± 0.22</td>
<td>-1.10</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>9.04 ± 0.26</td>
<td>-0.22</td>
</tr>
<tr>
<td>Anafranil 25 (tablets)</td>
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<td>5</td>
<td>24.06 ± 1.11</td>
<td>-1.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>23.46 ± 0.90</td>
<td>-3.30</td>
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<tr>
<td>Anafranil 75 (delayed-release tablets)</td>
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<td>5</td>
<td>69.79 ± 1.74</td>
<td>-1.70</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>70.42 ± 2.04</td>
<td>0.82</td>
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</table>

\(^a\) Relative deviation of the developed methodology with respect to the reference procedure.
The developed methodology was evaluated in the determination of clomipramine in pharmaceutical formulations. The obtained results when compared with those furnished by the reference method showed a relative deviation from −3.3 to 0.82%. The results are summarized in Table 3. The sampling frequency was about 19 samples per hour for a 10-s sampling time and 32 samples per hour at a 5-s sampling time.

4. Conclusion

An automated multicommutated flow system for the CL determination of clomipramine in pharmaceutical preparations was developed. The procedure was implemented using a single pumping channel for the insertion of all solutions, which facilitated system automation, operation and control, minimising the sources of eventual operational errors as well. Moreover, it enabled an effective manipulation of sample dispersion providing the means for the analysis of clomipramine samples in a wide range of concentration values. The synchronised insertion of time-based controlled reagent volumes at well-defined sections of the sample plug assure an efficient reaction zone formation and minimised reagent consumption.

The proposed method is simple, rapid, automate, sensitive and precise. The results obtained confirm the proposed methodology as a suitable alternative for the routine and control analysis of clomipramine in bulk drug and pharmaceutical preparations. This assumption is reinforced by the limited interference of the substances commonly used as excipients.

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References