Automated spectrophotometric determination of clomipramine on a multicommutated flow system

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Abstract
An automated multicommutated flow system for the spectrophotometric determination of clomipramine in pharmaceutical preparations was developed. The method was based on the oxidation of clomipramine by ammonium monovanadate in acidic medium yielding a coloured product with an absorbance maximum at 620 nm. The reaction development was enhanced when the binary sampling approach was exploited to insert the sample into the carrier stream. This approach, consisting of the intercalation of small sample and reagent aliquots, led to a more efficient sample zone homogenisation. The determinations were carried out at two distinct sampling times (6 and 12 s) in order to evaluate the system’s performance under different dispersion conditions, which also showed the versatility of the multicommutation time-based sample insertion.

Beer’s law was verified for clomipramine concentrations of up to 50 mg l\(^{-1}\). R.S.D. (n = 10) < 2.0 and 1.3% were attained for sampling times of 6 and 12 s, respectively. The results were in agreement with those obtained by the reference procedure, with R.D. < 2.6%.

Keywords: Clomipramine; Multicommutated flow system; Spectrophotometry

1. Introduction
Clomipramine hydrochloride, 3-chloro-5-[3-(dimethylamino)propyl]-10,11-dihydro-5H-dibenzo[b,f]azepine monohydrochloride (Fig. 1) is a dibenzoazepine derivative drug belonging to the class of pharmacological agents known as tricyclic antidepressants, which are commonly used in the treatment of the obsessive-compulsive perturbation states of phobia and panic as well as in depression and other emotional disturbances. The therapeutical and pharmacological importance of clomipramine has prompted the development of several methods for its determination, both in body fluids and pharmaceuticals, including liquid chromatographic methods with different detectors [1–3], gas chromatography [4], spectrophotometry [5,6] and chemiluminescence [7].

Dibenzoazepines have characteristic reactive structures conferred by the presence of chemically active nitrogen atoms readily partaking in distinct chemical reactions which could be used for analytical purposes. Preliminary experiments showed that clomipramine...
reacts with strong oxidants in an acidic medium forming coloured oxidation products, the products stability and the reaction kinetics being affected by the type and concentration of acid. This reaction could be easily implemented as a fast, simple and expeditious method for the determination of clomipramine in pharmaceutical preparations as it is readily adapted to routine analysis specially if combined with all of the analytical potentialities provided by automated multicommutated flow systems [8]. By combining a cluster of extremely advantageous operational characteristics, like flow manifold simplicity and functionality with a new sample insertion concept—binary sampling—multicommutation provide the means for fast reaction development, low reagent consumption and a wide variety of interventions over the sample zone without requiring modifications of the manifold configuration. These capacities could be further improved by both the utilisation of a time-based sampling approach, which enables the selection of the most appropriate sample volume, and the possible implementation of complementary analytical strategies, like zone sampling or sample re-circulation, that ensure effective control of sample dispersion and enable the attainment of a wide range of dilution levels which could be not only adjusted to the sample concentration but also provide an extended working range of determination.

In this work a multicommutated flow system that allows more efficient solution mixing and thus a faster reaction zone homogenisation is proposed for the spectrophotometric determination of clomipramine.

2. Experimental

2.1. Reagents

Reagents were all of analytical grade and doubly deionised water (conductivity < 0.1 µS cm⁻¹) was used throughout.

A 500 mg l⁻¹ clomipramine standard solution was daily prepared by dissolving 25 mg in 50 ml of 5.0 mol l⁻¹ sulphuric acid. This solution was kept in the refrigerator. Working standards were prepared by appropriately diluting the above solution with 5.0 mol l⁻¹ sulphuric acid.

A 0.1 mol l⁻¹ ammonium monovanadate stock solution was prepared by dissolving 0.585 g in 5.0 mol l⁻¹ sulphuric acid and completing to 50 cm³ with the same acid solution.

The solutions of commercially available pharmaceutical preparations (Anafranil tablets dosed 10 and 25 mg, Anafranil delayed releasing tablets dosed 75 mg and Anafranil injectable dosed 25 mg/2 ml) were prepared either by dissolving the required amounts of powdered tablets or the required volume of injectable solution with 5.0 mol l⁻¹ sulphuric acid and completing to 100 cm³ with the same acid solution. The sample solutions that were analysed by the developed procedure were not subject to any pre-treatment. When required they were filtered on-line. The sample solutions that were analysed by the reference method were manually filtered.

2.2. Equipment

A 6100 Jenway (UK) spectrophotometer equipped with an 18 µl inner volume flow-cell was used for absorbance measurements at 620 nm.

The flow manifold included two 161 T031 (NResearch, USA) three-way (two inlets and one outlet) solenoid valves. Flow lines and reaction coils were made from 0.8 mm i.d. PTFE tubing. Home-made end-fittings, connectors and confluence points were also used. Sample solutions were filtered on-line through a Schleicher & Schuell FM 0130 filter. A Crison Micro BU 2030 automatic burette equipped with a 5 ml syringe and controlled by a microcomputer through serial protocol (RS-232C) was used to propel the solutions by aspiration. A home-made power drive based on a UNL 2003 integrated circuit was used to operate the solenoid valves. Data acquisition and analytical system control was achieved by means of a PC-LABCard model PCL-818L interface card from Advantech and a 486DX-based microcomputer. The software was developed in BASIC and enabled the control of the solenoid valves position as well as
2.3. Flow manifold

The flow system (Fig. 2) was designed with two three-way solenoid valves, \( V_1 \) and \( V_2 \). \( V_1 \) was responsible for sample and reagent insertion and \( V_2 \) was used to select two distinct pathways during sample replacement and cleansing.

The carrier solution, which was also the reagent, was inserted through \( V_1 \) and was used to establish the baseline. The sample solution was also inserted through \( V_1 \) at a pre-set sampling time which, depending on the flow rate, established the sampling volume and ensured effective control of sample dispersion. By means of the exploitation of the binary sampling approach the sample was not inserted as a whole volume but as very small segments which were intercalated with very small reagent segments. The number and timing of each intercalation cycle defined the final sampling time. Initially, \( V_1 \) and \( V_2 \) were placed in position 1 and the carrier solution flowed through the detector. \( V_2 \) was then actuated repeatedly between positions 2 and 1, at pre-set intercalation times, allowing the insertion and intercalation of small sample and reagent aliquots which favoured reaction zone homogenisation and thus reaction development. \( V_1 \) was subsequently moved back to position 1 and the sample zone was carried through \( L_1 \) towards the detector. Following sample detection, \( V_1 \) and \( V_2 \) were actuated to position 2 for cleanup and sample replacement. Since the sample solutions were obtained by dissolution of the pharmaceutical forms with no other sample pre-treatment, and considering that some of the excipients were not soluble, a filtration had to be carried out. This filtration was executed on-line through a filter (F) placed prior to \( V_1 \).

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

2.4. Reference method

The accuracy of the developed procedure was evaluated by analysis of either clomipramine bulk drug and clomipramine pharmaceutical formulations according to the British Pharmacopoeia [9] by UV spectrophotometry at 252 nm. This procedure involves two consecutive dilutions of the sample and a filtration step.

3. Results and discussion

Preliminary batch studies to evaluate the effect of strong oxidants on clomipramine solutions revealed the formation of coloured compounds that exhibit an absorbance maximum at 620 nm. These results comply with other work available in the literature reporting that the mechanism of oxidation of dibenzoazepines involves the formation of an intense blue dimeric species [10]. Ammonium vanadate was selected as the oxidant because it produced stable oxidation products rapidly. Moreover, this reagent is easily obtainable and, as was later confirmed, its reaction with clomipramine does not require heating or a long time, which makes it very attractive for use in continuous flow methodology that is intended to be fast, sensitive and selective.

The oxidation reaction was enhanced in acidic media and from the first results it was evident that the reaction rate was affected by the type and concentration of the acid being used (Fig. 3). The influence of acid concentration was evaluated by inserting a 20 mg l\(^{-1}\) clomipramine solution prepared in nitric, sulphuric and hydrochloric acids at acid concentrations ranging from 2.0 to 6.0 mol l\(^{-1}\), which were also used to prepare the vanadate reagent solution. It was observed that for hydrochloric and sulphuric acids the analytical signal showed a slight increase as the acid concentration increased from 2.0 to 3.0 mol l\(^{-1}\), and a
very pronounced increase between 3.0 and 5.0 mol l\(^{-1}\). From 5.0 to 6.0 mol l\(^{-1}\) the signal approached stabilisation. For nitric acid the analytical signal exhibited a continuous but discrete increase throughout the acid concentration range evaluated. Moreover, it was observed that at acid concentrations < 3.3 mol l\(^{-1}\) the analytical signal obtained for nitric acid was higher than those obtained with sulphuric and hydrochloric acids, but this situation rapidly changed as the acid concentration reached higher values, where the latter acids caused markedly increased absorbance readings. This behaviour was due to faster reaction development in nitric acid along with a less stability of the oxidation products. Thus, as the acid concentration increased, the corresponding increase in the reaction rate in sulphuric and hydrochloric acids guaranteed that for the same reaction time colour development was maximised while in nitric acid it was already fading. The highest peaks were obtained when the oxidation of clomipramine was carried out in sulphuric acid 6.0 mol l\(^{-1}\) (Fig. 4). Taking into account that sulphuric acid solutions are viscous, which could be a drawback when the solutions are aspirated, and that the difference between the analytical signals obtained both for

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**Fig. 3.** Absorption spectra of clomipramine oxidation products obtained in 5.0 mol l\(^{-1}\) acid solutions: (a) H\(_2\)SO\(_4\); (b) HCl; (c) HNO\(_3\).

**Fig. 4.** Influence of sulphuric acid and ammonium vanadate concentrations: ■ sulphuric acid; ▲ ammonium vanadate.
5.0 and 6.0 mol·l⁻¹ H₂SO₄ was not so significant as to compromise the sensitivity of the determinations, a 5.0 mol·l⁻¹ sulphuric acid solution was selected for subsequent experiments.

An aspect of great importance was the strategy used for sample introduction. By inserting increasing volumes of a 10 mg·l⁻¹ clomipramine solution it was verified that the utilisation of a binary sampling approach consisting of the intercalation of very small aliquots of clomipramine solution with very small aliquots of monovanadate solution (creating a continuous string of sample/reagent interfaces) improved significantly the signal intensity [11]. This was due to the low dispersion string of interfaces that favoured clomipramine/vanadate homogenisation, thus enhancing reaction development. This approach was very important since the working solutions were prepared in a viscous 5.0 mol·l⁻¹ sulphuric acid solution. Because of the viscosity of the 5.0 mol·l⁻¹ sulphuric acid, a sample inserted as a single volume, giving rise to only two reaction interfaces, would restrict the reaction development due to the inadequate mixing between sample and reagent. Instead of using longer reaction coils to achieve efficient mixing, which would result in a subsequent increase in sample dispersion and decrease in peak height, the exploitation of the binary sampling approach provides multiple reaction interfaces that behave like a unique and homogeneous reaction zone while the analytical system remains simple and effective. The evaluation of the binary sampling performance was carried out by establishing a whole sampling time of 12 s (that for a 1.0 ml·min⁻¹ flow rate corresponded to a 200 µl sample volume) and by using distinct intercalation sequences in such a way as to maintain a constant value for the product number of cycles × intercalation time: 12 cycles × 1 s (for sample and reagent); 6 cycles × 2 s; 4 cycles × 3 s; 6 cycles × 2 s and 1 cycle × 12 s. The results confirmed that the utilisation of an intercalation time of 1 s enabled the highest peak to be obtained as a consequence of an improved sample/reagent mixing.

The influence of vanadate concentration was evaluated by comparing the analytical signals obtained with a 20 mg·l⁻¹ clomipramine solution when ammonium monovanadate solutions in the 1.0 × 10⁻⁴ to 8.0 × 10⁻⁴ mol·l⁻¹ range were used. It was shown (Fig. 4) that from 1.0 × 10⁻⁴ to 6.0 × 10⁻⁴ mol·l⁻¹ the peak height showed a pronounced increase and then it approached stabilisation.

The study of the influence of the reaction’s coil length and flow rate was carried out simultaneously since these parameters determine the residence time and thus the reaction extension, thus affecting the analytical signal magnitude. A 10 mg·l⁻¹ clomipramine solution was inserted at distinct sampling times and was carried toward the detector at flow rates of 0.5, 1.0 or 1.5 ml·min⁻¹. At the same time the length of the analytical pathway was changed and reaction coils of 0.5, 1.0 or 2 m were used for each of the referred flow rates. It was observed that for a flow rate of 0.5 ml·min⁻¹ the highest analytical signal was obtained with a 1 m reaction coil. Increasing the coil length resulted in a lesser peak height that could be explained by an increase in the sample dispersion which offset the simultaneous increase in the reaction time. A 0.5 m reactor produced analytical signals similar to those obtained with the 1 m reactor but only when sample volumes < 40 µl were used. As the sample volume increased the peak height decreased and approached the value obtained with the 2 m reactor. These results confirmed that large sample dispersions (with the 2 m reactor) and small reaction times or inefficient sample/reagent mixing (with the 0.5 m reactor) resulted in poorer analytical signals.

For a flow rate of 1.0 ml·min⁻¹ and sample volume < 166 µl the peak height for a 1 m reactor was similar to that obtained with the 1.5 m reactor but greater than that obtained with the 2 m reactor. However for larger sample volumes it was verified that the peak height increased with the coil length. Similar behaviour was found at 1.5 ml·min⁻¹ and all of the coil’s lengths tested. In this case, the dispersion was sufficient to guarantee an adequate sample/reagent mixing while the increasing reaction time provided extended colour development. In fact, reactors of reduced length resulted in a short residence time and when the sample arrived at the detector the reaction had not reached completion.

Considering that for a 1 m reactor the variation in the peaks height obtained both for flow rates of 1.0 and 0.5 ml·min⁻¹ was not significant and aiming to establish a compromise between sensitivity and sampling rate, a 1.0 ml·min⁻¹ flow rate was selected for subsequent experiments.
The evaluation of the sampling volume was carried out based on the sampling time ($t_s$), which for a given flow rate determined the sample aliquot volume introduced in the analytical system. It was verified that by controlling the sampling time it was possible to manipulate sample dispersion and thus the working concentration range, which constitutes one of the major advantages of multicommutation: the time-based unlimited variation of sample volume. This study was executed by inserting a 10 mg l$^{-1}$ clomipramine solution for increasing sampling times. For a 1 m reactor and a flow rate of 1.0 ml min$^{-1}$ it was observed that peak height increased with sampling time and that the maximum analytical signal was obtained with a $t_s$ of 12 s, that corresponded, approximately, to a sample volume of 200 $\mu$l. The absorbance value was almost twice the value obtained with a sampling time of 6 s (100 $\mu$l sampling volume). These results confirmed that without reconfiguring the analytical system it was possible to carry out the analysis at different sensitivities, which resulted in distinct working concentration ranges. In fact, a 6 s $t_s$ enabled the analysis of samples within a concentration range up to 50 mg l$^{-1}$ while with a 12 $t_s$ the maximum sample concentration that could be measured was 40 mg l$^{-1}$, but with enhanced sensitivity. The possibility of measuring distinct sample concentrations without requiring system reconfiguration could be an advantageous feature which could be particularly useful when applied in dissolution studies where the solution concentration could change from zero to high values.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration declared (mg per formulation)</th>
<th>Concentration found (mg per formulation)</th>
<th>$RD^a$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sampling time (s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Developed methodology$^b$</td>
<td>Reference method</td>
<td></td>
</tr>
<tr>
<td>Anafranil 10 (tablets)</td>
<td>10</td>
<td>10.1 ± 0.1</td>
<td>-1.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10.1 ± 0.1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>10.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Anafranil 25 (tablets)</td>
<td>25</td>
<td>25.4 ± 0.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>25.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>25.1 ± 0.3</td>
<td>-1.2</td>
</tr>
<tr>
<td>Anafranil 75 (delayed-release tablets)</td>
<td>75</td>
<td>74.2 ± 0.3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>74.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>75.5 ± 0.9</td>
<td>2.6</td>
</tr>
<tr>
<td>Anafranil 25 (injectable)</td>
<td>25</td>
<td>25.2 ± 0.5</td>
<td>-1.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>25.2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>25.7 ± 0.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$^a$ Relative deviation of the developed methodology with respect to the reference procedure.

With a comparison purpose and aiming to obtain a compromise between sampling rate and sensitivity, sampling times of 6 and 12 s were used to carry out the determinations. The results confirmed that for a continuous routine application a choice has to be made: a 6 s sampling time improves sampling rate but reduces the analytical signal while a 12 s sampling time maximises the analytical signal and thus the sensitivity of the determination but affects markedly the sampling rate.

3.1. Analysis of pharmaceutical preparations

After system optimisation with a flow rate of 1.0 ml min$^{-1}$ and a reaction coil of 1 m and by using sampling intercalation sequences of 6 and 12 cycles of 1 s sample/1 s ammonium monovanadate, linear working ranges for clomipramine concentrations up to 50 and 40 mg l$^{-1}$, respectively, were obtained. For a sampling time of 6 s, the analytical curve was represented as $A = 0.1904 C + 0.0665$ 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 0.9992. The R.S.D. was <2.0% ($n = 10$). For a 12 s sampling time, the equation was $A = 0.2903 C + 0.1496$ and the correlation coefficient was 0.9998. The R.S.D. was <1.3% ($n = 10$).

The developed methodology was evaluated for the determination of clomipramine in pharmaceutical formulations. The obtained results when compared with
those furnished by the reference method showed a relative deviation between $-1.2$ and $2.6\%$.

The analytical system was stable and there was no baseline drift. The sample throughput rate was about $15$ h$^{-1}$. The results are summarised in Table 1.

### 3.2. Interferences

In order to apply the developed methodology to the determination of clomipramine in pharmaceutical formulations, the influence of some compounds commonly used as excipients was assessed. Sample solutions containing a fixed amount of clomipramine ($20$ and $30$ mg l$^{-1}$) and different concentrations of the excipients under evaluation were analysed by the developed method. A compound was considered as non-interfering if the analytical signal variation was $\pm 3\%$ when compared to the analytical signal obtained in the absence of the referred compound. The results revealed that the excipients (glucose, sucrose, galactose, lactose, sodium benzoate and magnesium stearate) upon a 100-fold molar ratio with respect to clomipramine did not interfere.

### 4. Conclusions

The results obtained showed that the spectrophotometric monitoring of the reaction products between clomipramine and vanadate is a valuable analytical strategy for the determination of this antidepressant drug in pharmaceutical preparations. When implemented in a continuous multicommutated flow system it allows the development of a simple, fast and easily automated methodology, which could be advantageously applied in routine analyses. This assumption is reinforced by the limited interference of the substances commonly used as excipients. Moreover, it does not require any sample pre-treatment (filtration is carried out on-line), as happens, for instance, with the reference method, which could noticeably improve the time required for the analysis. Despite its simplicity the proposed single-channel flow methodology combined with fast homogeneous mixing achieved with the binary sampling with an effective control of dispersion, due to the versatile time-based sample insertion and low reagent consumption. Moreover, it proved to be accurate and versatile because it enables multiple sample manipulations without requiring re-configuration. On the other hand, due to its modular structure, when necessary it can easily be modified for application to other compounds.

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### References