1. Introduction

The use of nitroheterocyclic drugs in the treatment of diverse pathologies caused by bacteria, protozoa and hypoxic human cells has been widely documented [1–6]. The reduction of the nitro group is a prerequisite for all biological activity [7, 8] and the first step, with formation of the nitro radical anion ($\text{ArNO}_2^-$), is crucial for the activity of these compounds. In fact, it has been affirmed [3] that the parameter that defines the reduction parameter of the couple $\text{ArNO}_2^- / \text{ArNO}_2$ is a very appropriate index to define the type of biological properties of the different nitrocompound. Studies carried out by Olive [9] on a series of nitroheterocyclic compounds demonstrated the existence of direct correlation among the polarographic half-wave potential ($E_{1/2}$) and the nitroreduction speed in different biological systems. In that work it concludes that the drugs with higher electroaffinity (smaller reduction potential) are generally the most toxic and mutagenic, being also characterized to be those metabolized more quickly. Furthermore there are a lot of other studies [10–12] dealing with the relationship between reduction potential and pharmacological activity showing that these measurements are of interest from electrochemical and pharmacological points of view.

The Chagas disease (American trypanosomiasis) caused by the pathogen Trypanosoma cruzi, is one of the many parasitic illnesses existing today, for which it has not been possible to find a satisfactory pharmacological treatment, thus constituting a serious problem of public health at world level [13]. The nitroheterocyclic drugs nifurtimox and benznidazol, are the only drugs that are used at the present time to combat this pathogen. However, due to their lateral effects in the host and also because their use has led to parasitic resistance, they will be retired from the market as soon as possible. For this reason, there is an urgent necessity to develop new drugs that could replace the existing ones. One example of new investigations are the studies with megazol (1-methyl-2-(5-amino-1,3,4-thiadiazole)-5-nitroimidazole) and related compounds (Fig. 1), which are being tested as antichagasic drugs.

Megazol, was synthesized in the sixties [14] and was shown to have a great antiprotozoan and antibacterial activity [15]. However, only recently, studies of this molecule have been reconsidered since it may be a viable alternative to the nifurtimox and benznidazol. Several authors demonstrated a high efficiency of megazol against Trypanosoma cruzi [16, 17]. Others have also demonstrated that this compound is highly active against Trypanosoma brucei [18, 19] and its pharmacokinetic, metabolism and excretion in animals have been published [20, 21]. In consequence, megazol has become a core structure for the design of new compounds and for the identification of biological target involved in both types of trypanosomiasis [22]. The exact nature of its pharmacological mode of action is unknown but there is strong evidence that the reduction of megazol is the key in that mechanism. The single electron reduction of megazol by NADPH:cytochrome P-450 reductase, by rat liver as well as by trypanosome microsomes was confirmed by ESR experiments [23].

In view of the similarity between the electrochemical reactions (at the electrode-solution interface) and the enzymatic reactions, the knowledge of the mechanism of electroreduction of these compounds could be a very good argument to provide additional information on the mechanism of action. Consequently we have focused our research to investigate the polarographic behavior of megazol and a series of related compounds. A survey of the literature reveals that no attempt has been made to study the polarographic behavior of megazol. However, several reports pertaining to the polarographic behavior of different
5-nitroimidazoles are available [24–28]. In this work, we studied by TAST and differential pulse polarography (DPP), the electrochemical behavior of megazol, related compounds and benznidazole in buffered aqueous media and in dimethylformamide (DMF) + buffer aqueous mixtures.

There appears to be only one HPLC method [29] described in the literature to analyze megazol. Furthermore for comparative analytical purposes we have also developed spectrophotometric and HPLC methods.

2. Experimental

2.1. Reagents and Solutions

All the compounds were synthesized and characterized by one of us according to a published procedure [30]. Benznidazole was commercially obtained from Laboratorios ROCHE Quimicos e Farmaceuticos S.A., Brasil. All the other reagents employed were of analytical grade.

Stock solutions of each compound were prepared at a constant concentration of 0.025 M in DMF. The polarographic working solutions were prepared by diluting the stock solution until to obtain final concentrations of 0.1 or 0.5 mM in a mixture of 30/70 : ethanol/Britton-Robinson buffer (KCl 0.3 M) for a protic medium and a mixture 60/40 : DMF/citrate buffer (KCl 0.3 M) for a mixed medium. The pH was adjusted with little aliquots of concentrated NaOH or HCl, respectively.

In mixed medium the pH measurements were corrected according to the following equation [31]:

$$\text{pH}^* - B = \log U_{\text{H}}^\phi$$

where pH* equals -log $a_{\text{H}}$ in the mixed solvent, B is the pH meter reading and the term log $U_{\text{H}}^\phi$ is the correction factor for the glass electrode, which was calculated from the different mixtures of DMF and aqueous solvent, according to a previously reported procedure [32].

Chronocoulometric measurements were conducted using a glassy carbon electrode. A 0.5 mM of each compound in protic medium (30/70 : ethanol/Britton-Robinson buffer (KCl 0.3 M)) at pH 7 were the selected experimental conditions for the solutions. The initial potential, where no electrolysis occurs, and final potential, where complete electrolysis occurs, were obtained from each cyclic voltammogram. The potentials chosen were 0 V and $-0.6 \ V$, respectively. The chronocoulometric response is the total charge passed ($Q_t$), vs. time ($t$). The response is described by

$$Q_t = (2nFACD^{1/2}t^{1/2})/\Gamma + Q_{di} + nFA\Gamma_o$$

where $Q_{di}$ is the capacitive charge, $\Gamma_o$ is the surface excess of reactant, and the others terms have their usual meaning.

A plot $Q_t$ vs. $t^{1/2}$ transform the data into a linear relationship whose slope permits the diffusion coefficient determination.

2.2. Apparatus

Electrochemical experiments, differential pulse polarography (DPP), TAST polarography were performed with a totally automated BAS CV-50W voltammetric analyzer. A 10 mL thermostated three electrode measuring cell, comprised of a dropping mercury electrode as a working electrode, a platinum wire as a counter electrode, and a Ag/AgCl as a reference electrode, were used for the measurements. Spectrophotometric measurements were carried out with a UV-vis spectrophotometer ATI Unicam Model UV3, using 1 cm quartz cell and equipped with a 486 computer with Vision acquisition and treatment program.

HPLC measurements were carried out using a Waters assembly equipped with a Model 600 controller pump and a Model 996
photodiode array detector. The acquisition and treatment of data were made by means of the Millenium 32 software. As chromatographic column a μBondapack/μPorasil C-18 column of 3.9 mm × 300 mm was used. As precolumn a C18 μBondapack (30 mm × 4.6 mm) was employed. The injector was a 20 μL Rheodyne valve.

2.3. Analytical Studies

For calibration plots, a series of ten solutions were prepared containing megazol concentration ranging between 2 × 10⁻³ M and 1.6 × 10⁻⁴ M in 30/70 : ethanol/Britton-Robinson buffer (KCl 0.3 M) at pH 6.

Recovery studies were conducted by DPP, spectrophotometry and HPLC techniques. A serie of ten synthetic samples were prepared by weighing 5 mg of megazol which were dissolved and diluted in the same conditions used for the calibration plot experiments, obtaining a final megazol concentration of 5 × 10⁻⁶ M.

2.3.1. Polarography

Ten sample solutions prepared in the same conditions of that for the solutions for the calibration plot, were transferred to a polarographic cell, degassed with nitrogen for 5 min and recorded at least twice from −100 to −600 mV. The content (mg amount) of megazol in the sample solution was calculated from a prepared standard calibration plot.

2.3.2. Spectrophotometry

The same samples used for polarography were transferred to a cuvette and were measured at 356 nm, and the content of megazol in the sample solution was calculated from a prepared standard calibration plot.

2.3.3. HPLC

An aliquot of the solutions prepared for polarography and spectrophotometry was taken and a 20 μL volume was injected into the chromatographic system. The mobile phase flow (methanol/0.05 M phosphate buffer (pH 3) : 30/70) was maintained at 1 mL/min and helium sparging (10 mL/min) was applied to remove dissolved gases. The temperature was kept constant at 35 °C.

3. Results and Discussion

All compounds were electrochemically reduced at the dropping mercury electrode (DME) in protic and mixed media. According to the molecular structures (Fig. 1), the most probable easily electroreducible group in all the molecules is the nitro group.

Considering that the reduction of the nitro group plays an important role in the biological activity of these compounds we were interested to study the influence of structural changes in the molecules on the reduction ability of the nitro group.

3.1. Protic Medium

In ethanol : aqueous Britton Robinson buffer solution (30:70), all the compounds were polarographically reduced at the DME producing two waves or peaks. Figure 2 shows the differential pulse (DPP) and TAST polarograms (TP) for all compounds. From this figure we can distinguish a rather different behavior depending on the molecular structure. All the compounds produce a main signal (I) (around −100 mV at pH 4) and another signal (II) at more negative potentials (around −1000 mV). The waves (I) have approximately the same intensity for all compounds and may arise as a result of the four-electron reduction of the nitro group to the hydroxylamine derivative. We have confirmed this assumption by comparison of the height of wave (I) for megazol with the corresponding nitroreduction wave of well studied nitroimidazole derivatives such us tinidazole and metronidazole [25, 28]. In the case of peak or wave (II) we observe a different behavior depending on the structure of the nitroimidazole derivative. For compounds RO150216 and benznidazole we can observe that the limiting current ratio between waves (I) and (II) are close to 2 claiming the typical published behavior for nitroimidazole derivatives wherein the wave (I) is due the four-electron reduction of the nitro group to the hydroxylamine derivative and the wave (II) is due to the subsequent two-electron reduction of the latter group to amino group [24–28]. In the case of megazol, GC-361, GC-360 and GC-284 (nitroimidazole-diazole derivatives) the behavior is rather different, wherein the limiting current ratio between both waves is close to 1 and consequently the wave (II) can be ascribed to the reduction of the diazole moiety.

In order to study the pH dependence of both waves we have obtained the polarograms by DPP and TP at different pHs for all the compounds. We have used the peak potential (E_p) obtained by DPP and to study the pH-current dependence we have used the limiting current (i_l) obtained by TP. The E_p vs. pH plots are shown in Figure 3. The potential of peak (I) was shifted to more negative values by raising the pH for all the studied compounds. The nitroimidazole-thiadiazole compounds (Megazol, GC-360, GC-361) show a linear behavior in all the pH range. However, in the case of benznidazole and GC-284 it is possible to observe a break in the E_p vs. pH curve at approximately pH 8.5. Above this value the process was pH-independent showing that no protonation occurs before or in the rate determining step. The slope of the linear portion for peak (I) is shown in Table 1. The E_p vs. pH plot for peak (II) shows substantial differences between the nitroimidazole-diazole compounds and the others, confirming that this second peak is due to a different process. In the case of the nitroimidazole-diazole compounds the reaction is likely due to the reduction of the thiadiazole or oxadiazole moieties showing a strong dependence with pH, while in the case of benznidazole and RO-150216 the reaction is due to the reduction of the hydroxylamine showing a pH-independency.

In Table 1 are summarized the peak potential values for the nitro reduction (peak I) for all the compounds at four different pHs. From this table we can conclude that GC-361 and GC-360, show similar values for peak potentials than megazol, indicating that the nitro group in this compounds has similar electron affinity. Consequently, this fact implies that the substitution on the amino group of the thiadiazole ring is not affecting the electron density on the nitro group. However, these compounds have strong differences in lipophilicity probably affecting their biodisponibility. This result is very interesting because it permits one to design new synthetic strategies for producing compounds with strong differences in lipophilicity without affecting the reduction ability of the nitro group. On the other hand, by comparing GC-284 with megazol we found that the replacement of the S atom in the thiadiazole group by an O atom produced a significant decrease in the electroreduction availability. This
effect can be ascribed by the electron donating character of the oxadiazole ring which increases the electron density on the nitro group. By comparing megazol and benznidazole we found that the former is significantly more readily reduced. Considering that benznidazole is actually used as a drug against the Trypanosoma cruzi and based on its nitroreduction ability, we may expect that megazol would be even a more effective drug. Also from the results in Table 1 we can deduce that, in all pH conditions, the RO-150216 compound was the most easily reduced indicating that the substitution in position 2 with a CH₂OR group produces a higher withdrawing effect than the thiadiazole or oxadiazole rings. Consequently, from this result we can deduce that it is possible to calibrate by DPP, the electron affinity power of the nitro group with a substituent with different electrodonor or withdrawing effects on the nitroimidazole ring.

In Figure 4 is shown the pH-dependence of the limiting current of the nitroreduction wave. The current values were pH-independent for all compounds, indicating that the limiting current was diffusion controlled. In the case of compounds GC-360 and GC-361 we observed a decrease in the limiting current due to solubility problems in nonacidic pHs. Furthermore by using the chronocoulometric technique on a glassy carbon electrode, we calculated the diffusion coefficient for each compound (Table 1). For all the nitroimidazole-diazole compounds the limiting current in acidic media was similar around 0.6 mA indicating that the molecules follow the same nitroreduction process, i.e., according to the well-known equation for nitroaromatic compounds [33]:

\[
\text{ArNO}_2 + 4e^- + 4\text{H}^+ \rightarrow \text{ArNHOH} + \text{H}_2\text{O} \quad (1)
\]

3.2. Mixed Medium
We have also studied the polarographic reduction of all the compounds in a mixed medium containing DMF/buffer...
citrate: 60/40 in all the apparent pH range. The compounds benznidazole and RO-150216 show a similar behavior as protic media but the nitroimidazole-diazole compounds show a rather different behavior. In Figure 5 we can observe the behavior of megazol compared with benznidazole. In the case of nitroimidazole-diazole compounds the limiting current due to nitro reduction was pH-independent until pH 7, showing a diffusion-controlled behavior (peak I). Above pH 7, the limiting current

Table 1. Peak potential values for the nitro reduction (peak I) of megazol and related compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$E_p$ (mV)</th>
<th>$D \times 10^6$ (cm$^2$/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3</td>
<td>pH 6</td>
<td>pH 9</td>
</tr>
<tr>
<td>Megazol</td>
<td>$-60$</td>
<td>$-222$</td>
</tr>
<tr>
<td>GC 361</td>
<td>$-54$</td>
<td>$-255$</td>
</tr>
<tr>
<td>GC 360</td>
<td>$-56$</td>
<td>$-244$</td>
</tr>
<tr>
<td>GC 284</td>
<td>$-126$</td>
<td>$-404$</td>
</tr>
<tr>
<td>RO150216</td>
<td>$-34$</td>
<td>$-210$</td>
</tr>
<tr>
<td>Benznidazol</td>
<td>$-62$</td>
<td>$-322$</td>
</tr>
</tbody>
</table>

[a] Slope of $E_p$ vs. pH curve for peak I.
[b] Diffusion coefficient obtained by chronocoulometry in protic media.

Fig. 3. Peak potential dependence of megazol and related compounds with pH.
decreased by raising the pH. Concomitant with this decrease, a new wave (I') is observed. The limiting current of this new wave increased with the pH. The sum of the limiting current for both waves (peak I and I') is comparable to the initial limiting current, showing that this effect is due to a splitting of the initial wave. Furthermore the limiting current ratio I:I' was 1:3. This behavior is in accordance with the following well-known mechanism for the reduction of nitroaromatic compounds [33, 34]:

\[
\text{At pH } < 7 \\
\text{ArNO}_2 + 4e^- + 4\text{H}^+ \rightarrow \text{ArNHOH} + \text{H}_2\text{O} \tag{2}
\]

At pH > 7

\[
\text{ArNO}_2^- + 3e^- + 4\text{H}^+ \rightarrow \text{ArNHOH} + \text{H}_2\text{O} \tag{4}
\]

From the above results we can conclude that in the case of the megazol and the related nitroimidazole-diazole compounds the nitro radical anion was sufficiently stabilized to produce a separate wave or peak. However, in the case of the 2-nitroimidazole compounds it was not possible to stabilize the nitro radical anion. This fact has considerable importance, because for conditions where the nitro radical anion of benznidazole (that is currently used as an antiprotozoal drug) was not stabilized, the nitro radical anion of megazol and its related compounds was stabilized.

3.3. UV-vis Spectrophotometric Characterization

With the aim of making a comparison between different techniques and to further explore the knowledge of the chemistry in solution of megazol, a UV-vis spectrophotometric study was conducted. UV-vis spectra at different pHs (Fig. 6A) reveal two absorption signals for megazol (356 nm and 276 nm) showing a slight pH-dependence. The sensitivity of the band at 276 nm with pH was used to determine the spectrophotometric apparent pK\(_a\) (Fig. 6B and C). The calculated pK\(_a\) was 8.5 and it corresponds to the protonation of the amine group substituent in the thia-diazole ring.

3.4. HPLC Characterization

According to the above spectrophotometric behavior it was possible to develop a HPLC method using spectrophotometric detection at 356 nm. In Figure 7 we can observe chromatograms of megazol at different concentrations showing a retention time of 6.3 minutes.
3.5. Analytical Studies

According to the obtained results by polarographic, spectrophotometric and chromatographic studies, it was possible to apply these techniques to the quantitative analysis of megazol. As working pH, pH 6 was selected for DPP and UV techniques and pH 3 (mobile phase = methanol/0.05 M phosphate buffer, pH 3): 30/70 for HPLC.

In order to provide a DPP quantitative procedure, the dependence between the megazol concentration and peak current ($I_p$) was conducted. The linearity was maintained between $6 \times 10^{-6}$ and $1 \times 10^{-5}$ M with a quantitation and detection limits of $6.7 \times 10^{-6}$ and $3.2 \times 10^{-6}$ M, respectively. For quantitation we have used the calibration plot method. The regression equation for the calibration plot for concentrations ranging between $2 \times 10^{-5}$ and $1.6 \times 10^{-4}$ M was:

$$DPP \: I_p \ (\mu A) = (8.1 \pm 0.13) \times C \ (M) - 4.37 \times 10^{-5}$$

$$r = 0.999, \ n = 10$$

On the other hand, with the UV spectrophotometric technique the concentration study was made at two absorbance bands (356 and 276) and showed different linear response ranges and sensitivity. We selected the 356 nm band because it has a greater sensitivity and linearity than the other. The linearity was maintained between $4.1 \times 10^{-6}$ M and $1 \times 10^{-5}$ M with a quantitation and detection limits of $4.1 \times 10^{-6}$ and $2.0 \times 10^{-6}$ M, respectively. For quantitation we have used the calibration plot method using concentrations ranging between $2 \times 10^{-5}$ M and $1.6 \times 10^{-4}$ M. The regression equation was:

$$UV-\text{vis} \ ABS = (11 \ 030 \pm 246) \times C \ (M) - 0.01$$

$$r = 0.999, \ n = 10$$

Furthermore we have used this band at 356 nm to detect the megazol in the HPLC separation. The chromatographic peak area obtained also shows a linear relation with the megazol concentration. The linearity was maintained between $5.5 \times 10^{-6}$ and $1 \times 10^{-4}$ M with a quantitation and detection limits of $5.5 \times 10^{-6}$ and $1.6 \times 10^{-6}$ M, respectively. The regression equation was:

$$HPLC \ A = (1.27 \times 10^{10} \pm 3.07 \times 10^8) \times C \ (M) - 4936$$

$$r = 0.998, \ n = 10$$

The repeatability of the methods was adequate with variation coefficients of 1.4%, 0.4% and 3.1% for ten DPP, UV-vis and HPLC measurements, respectively. In order to obtain the precision and accuracy of the developed methods, we performed a recovery study for all the techniques. The results are summarized in Table 2. These results reveal that all methods had adequate precision and accuracy and consequently can be applied to the determination of megazol. In spite of at this moment pharmaceutical formulations of megazol are not yet developed we have tested common excipients such as cornstarch, magnesium stearate, lactose and talc showing that the excipients do not interfere in the determination. In conclusion we propose this developed polarographic method for future applications to capsules or tablets containing megazol.

4. Acknowledgements

The authors gratefully acknowledge the financial support of FONDECYT Grant 8000016 and DID-University of Chile Grant No. I004-98/2. Also the financial support from ECOS-Connicyt for travel expenses of Dr. Chauviere and Dra. Bollo is recognized.

5. References