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Study on the electrochemical detection of the macrolide antibiotics clarithromycin and roxithromycin in reversed-phase high-performance liquid chromatography

A. Pappa-Louisi[a,*], A. Papageorgiou[a], A. Zitrou[a], S. Sotiropoulos[a], E. Georgarakis[b], F. Zougrou[b]

[a]*Laboratory of Physical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, 54006 Thessaloniki, Greece*
[b]*Section of Pharmaceutics and Drug Control, Department of Pharmacy, Aristotle University of Thessaloniki, 54006 Thessaloniki, Greece*

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Abstract

The optimal conditions of the amperometric detection of the macrolide antibiotics clarithromycin and roxithromycin were found by cyclic voltammetric studies and HPLC–electrochemical detection responses obtained in different temperatures (25.5–60°C) and different but almost isoelutropic binary, ternary and quaternary mixtures of aqueous buffer (pH 7), methanol, acetonitrile and isopropanol. These conditions were also proved to be applicable for the quantitative detection of clarithromycin in human plasma using roxithromycin as an internal standard and vice versa. It was demonstrated that increased attention has to be paid to eluent composition and column temperature to ensure sensitive and reproducible electrochemical responses as well as regularly shaped peaks for both macrolides tested.

Keywords: Clarithromycin; Roxithromycin

1. Introduction

Clarithromycin (Clari) and roxithromycin (Roxi) are relatively new semisynthetic macrolide antibiotics derived from erythromycin A and consist of a 14-membered macrocyclic lactone ring with sugars linked via glycosidic bonds. All macrolides display similar antibacterial properties and constitute an important alternative for patients exhibiting penicillin sensitivity and allergy [1]. Moreover, Clari and Roxi are more stable to the acid environment of the stomach and they exhibit better oral bioavailability and a more favorable pharmacokinetic behavior [2]. This efficacy can be enhanced by a suitable method for measuring Clari accurately and rapidly, using Roxi as an internal standard and vice versa in biological fluids, thereby ensuring that bactericidal levels are achieved and maintained. So far, a limited number of papers can be found in literature concerning the analysis of Clari and Roxi in biomatrices [2–12], whereas the documentation of erythromycin is more extensive. However, no paper has sys-

*Corresponding author. Tel.: +30-31-997-765; fax: +30-31-997-709.

E-mail address: apappa@chem.auth.gr (A. Pappa-Louisi).

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tematically examined the retention and detection of these and other related macrolides under different chromatographic conditions to date.

It is generally agreed that a step-by-step optimization procedure of any reliable chromatographic assay requires a good resolution with acceptable individual retention times without interfering background peaks and a good and stable sensitivity without peak tailing. Until now we have studied extensively the retention behavior of Clari and Roxi under varying mobile phase composition and column temperature [13]. The aim of the present study is to explore the effects of the nature of organic modifier and column temperature on the electrochemical detection (ED) response (peak area and peak shape) as well as on the loss of sensitivity, in order to find out the optimal conditions for assay of these drugs. Take into account that ED has proved to be the most appropriate mode for monitoring macrolides because of the poor UV absorbance of these substances and the need for derivatization prior to liquid chromatography when using fluorescence detection [1].

2. Experimental

2.1. Chromatographic system and conditions

The liquid chromatography system consisted of a Shimadzu LC-9A pump, a Model 7125 syringe loading manual sample injector (Rheodyne, Cotati, CA, USA), a 250×4 mm MZ-Analytical column (5 μm Inertsil ODS-3) thermostatted by a 1270 temperature control system (Chemical Electronics) and a Gilson ED system (Model 141) equipped with a small disc (3 mm diameter) glassy carbon electrode. The detection of the analytes was performed at 1.0 V vs. the Ag/AgCl reference electrode, unless otherwise mentioned. The detector was interfaced to a Pentium personal computer (at 200 MHz) via a 14-bit AD-DA card. In some cases a Shimadzu UV–visible spectrophotometric detector (Model SPD-10A) was connected in series with the ED system so that the analytes separated on the high-performance liquid chromatography (HPLC) column flowed first through the UV detector and then through the ED system. This allowed dual measurement of analytes by UV absorbance and oxidation response at the ED system. Since the column temperature as well as the mobile phase composition was systematically varied in this work (see below), the column was equilibrated with the new eluent at each temperature for at least 30 min before any measurement was made. Experiments were performed over the range of temperature from 25.5 to 60°C (±0.1°C). The volume flow-rate of all mobile phases used was 1.0 ml/min. A volume of 20–100 μl of standard solutions was injected.

2.2. Chemicals, standard solutions and mobile phases

All chemicals were used as received from commercial sources. Clari and Roxi were provided by Pharmanel, Pharma Industry (Athens, Greece). All other reagents were of analytical grade and solvents were of HPLC grade.

The macrolide antibiotics were dissolved to a concentration of 100 μg/ml in methanol (MeOH) and/or acetonitrile (ACN). Working solutions of standards (0.05–10 μg/ml) were made by an appropriate dilution of the stock solutions in aqueous phosphate buffer of pH 7 and ionic strength $I=0.02$ *M*. Solutes were injected individually or together using appropriate mixtures. The solute retention times and peak areas were measured successively several times (at least three runs) under identical conditions. Such data were averaged and will be referred to as a single data point. All solutions were kept refrigerated at 4°C when not in use.

Binary, ternary and quaternary eluent systems were used. The different mobile phases with constant pH value equal to 7 and ionic strength 0.02 *M* consisted of an aqueous phosphate buffer and one, two or three of the following organic modifiers MeOH, ACN and isopropanol (iPrOH). The compositions of the mobile phases used in this investigation are specified in Table 1. All the eluents were filtered through a mixed esters membrane filter (0.45 μm; Schleicher & Schuell, Germany), sonicated and degassed under vacuum for 5 min before use. The mobile phases were recycled into a 1-l reservoir.

2.3. Cyclic voltammetry

Cyclic voltammetry (CV) of Clari and Roxi was conducted on a laboratory-made cyclic voltammograph equipped with a glassy carbon small disc (3 mm

Table 1
Influence of organic modifier on quantitative parameters of 100 μl injected of 1 μg/ml Clari[a]

Organic modifier content in the mobile phases	Retention time (min)	Peak integral (nC)	Peak asymmetry
80% MeOH	12.4	282	1.00
70% MeOH–iPrOH (6:1, v/v)	15.3	298	1.01
28% iPrOH	26.2	321	1.42
70% MeOH–ACN–iPrOH (3:3:1, v/v)	13.8	375	1.05
75% MeOH–ACN (3:4, v/v)	16.3	429	0.95
55% ACN–iPrOH (6:1, v/v)	15.2	437	1.20
50% ACN	20.8	453	1.67

[a] Column temperature: 40°C.

diameter) or a microdisc carbon working electrode (30 μm diameter) and a Ag/AgCl reference electrode. Compounds were dissolved in the corresponding mobile phases used in HPLC to give concentrations in the range from 0.15 to 0.9 m*M*. Current was recorded over a voltage range of −0.5 to 1.3 V at a scan rate of 10 and/or 100 mV/s. Experiments were performed over the same range of temperature from 25.5 to 60°C.

2.4. Sample preparation and solid-phase extraction

Plasma samples were prepared by mixing 500 μl of drug-free plasma, 500 μl of a solution containing 1 μg/ml of both Clari and Roxi in phosphate buffer, pH 7 (I=0.02) with 80% MeOH and 1 ml ACN. After vortex mixing, the samples were centrifuged in order to precipitate plasma proteins. The supernatant was transferred to the extraction column after evaporation of the organic solvent.

The solid-phase extraction (SPE) procedure is summarized as follows: (a) a series of packing materials of cartridges from different manufacturers were investigated, Alltech C_8, Supelco, Baker and/or Rigas Labs C_{18}, Waters Oasis HLB. (b) A first step of cartridge activation with 2×3 ml MeOH and a second step with 2×3 ml of water or a phosphate buffer of pH 7 and/or pH 10.6 were tested. (c) The plasma matrix was wasted off the cartridges using water or aqueous buffer of pH 7 and/or pH 10.6. (d) The drugs were eluted with MeOH, the organic solution was evaporated to dryness and reconstituted in 500 μl of the mobile phase consisting of a phosphate buffer, pH 7 and 80% MeOH. (e) Aliquots of 50 μl were injected onto the analytical column at 40°C using mobile phase of pH 7 and 80% MeOH.

3. Results and discussion

3.1. Electrochemical detection

Macrolide antibiotics like Clari and Roxi, which contain a tertiary amino group, are detectable by electrochemical oxidation [4]. The optimal detector cell potential for the oxidation of these antibiotics has been investigated previously. Hydrodynamic voltammograms showed that a working potential of 0.85 to 1.1 V was needed for maximum sensitivity [4,11]. This was confirmed for our instrumentation, using a mobile phase with 80% MeOH and pH 7 at 25.5°C. In addition, cyclic voltammograms of Clari obtained in 80% MeOH under alkaline conditions (pH ≥7) showed a decrease of potential required for oxidation of Clari with increasing pH. For example, the cyclic voltammetry of Clari at pH 7.0, 8.0, 9.1, 10.0 and 11.9 showed an anodic wave with $E_{p/2}$ at 0.80, 0.76, 0.75, 0.66 and 0.64 V, respectively, being consistent with the results of other investigators, who indicated that the electrochemical oxidation of macrolides in unprotonated form was facilitated compared with their oxidation in protonated form [14]. However, a mobile phase pH above the pK_a of a basic solute results in its retention increase as well as in peak broadening and peak tailing [15]. Moreover, a conventional silica-based C_{18} column, such as that used in this study, is not stable under alkaline conditions. Therefore, our study was restricted at pH 7 and the amperometric cell potential was set at 1.0 V vs. the reference electrode. At this high potential, however, an ED system with dual coulometric electrodes appears to be the most advantageous [2–4,7,9], although single-channel amperometric detectors have been used [8,11]. The limitations of the

existing chromatographic methods with coulometric and mainly with amperometric detection include progressive passivation of the working electrode, which, in our opinion, as well as detection sensitivity and peak shape have the potential to be improved by choosing appropriate chromatographic conditions, i.e., appropriate eluent system and column temperature. The results of our systematic study concerning the effects of mobile phase composition and column temperature on amperometric detection of Clari and Roxi follow.

3.1.1. Influence of organic modifiers on the ED response and peak shape

Table 1 shows the manner in which ED response and peak tailing of Clari vary under changing mobile phase conditions. The organic modifier concentrations in binary, ternary and quaternary mobile phases were chosen in order to obtain retention times of each solute usually applicable to optimization procedures except in case a binary mobile phase with iPrOH was used, where a weak percentage of iPrOH was necessary because of the excessively high column pressure obtained with iPrOH volume fraction higher than 0.28.

Our results show that striking differences in peak area and shape existed between the different modifiers. For the solutes studied, detection sensitivity was least in the binary mobile phase with MeOH and greatest when ACN was used instead of MeOH. In addition, ACN led to an increase of peak asymmetry for both macrolides tested, see also Fig. 1. However, for a particular mobile phase it was found that by increasing the organic modifier content the peak shape was almost unaffected, but the ED response for both solutes decreased considerably.

The dependence of ED response on the nature of organic modifier was also shown in cyclic voltammograms obtained using a glassy carbon small disc (3 mm diameter) as well as a microdisc carbon working electrode (30 μm diameter), Fig. 2.

Increased attention has to be paid to the stability of ED response. Thus, the influence of mobile phase composition on the passivation of the ED electrode, which is a glassy carbon small disc electrode, was examined in order to ensure reproducible responses for both macrolides tested. Fig. 3 shows that, for example when the eluent system with 50% ACN was

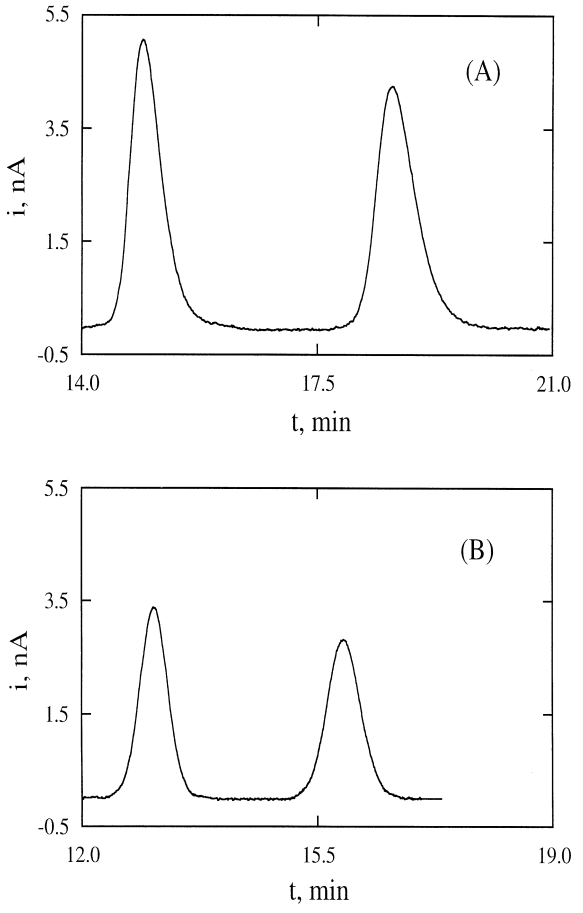

Fig. 1. Chromatograms of 50 μl of a mixture containing 1 μg/ml of Clari (the former peak) and Roxi (the latter peak) at 30°C using different mobile phases: 50% ACN (A) and 80% MeOH (B). See text for other chromatographic conditions.

used, repeated injections of a mixture of Clari and Roxi caused rapid loss of sensitivity due to electrode passivation. Detector sensitivity decreased at a rate of 50% after 13 injections within a working day. Therefore, the use of an internal standard is mandatory at least with mobile phase containing ACN. In contrast the deterioration was not rapid when the mobile phase with 80% MeOH was used, see Fig. 3. In this case decrease of ED response was very gradual and repeated injections of Clari and Roxi could be made over a period of 15 days before re-polishing of the electrode was necessary. In addition, there is an indication that poor peak shape and loss in ED sensitivity are related, since it was

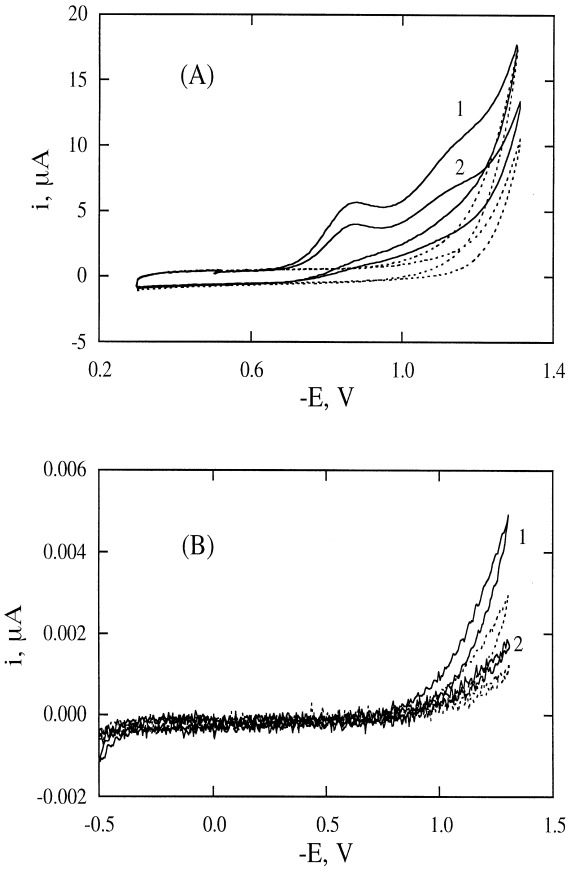

Fig. 2. Cyclic voltammograms of 0.5 m*M* Clari using a macro (A) and a micro (B) electrode in 75% MeOH–ACN (1) and in 70% MeOH–iPrOH (2). Background cyclic voltammograms are shown as dotted lines.

found that the higher the observed peak asymmetry in an eluent system, the higher the progressive poisoning of the working electrode.

In conclusion, the eluent system was found to have a crucial effect on peak shape, ED sensitivity as well as on sensitivity loss. In contrast, the UV absorbance of these compounds, although weak, obtained only at wavelengths ≤210 nm and associated with considerable background noise [1], was found to be stable and independent of the eluent system used. As a result, UV detection at 210 nm in series with ED can be useful if electrode passivation occurs.

Moreover, in any case the loss in detector sensitivity was more pronounced for Roxi than for Clari,

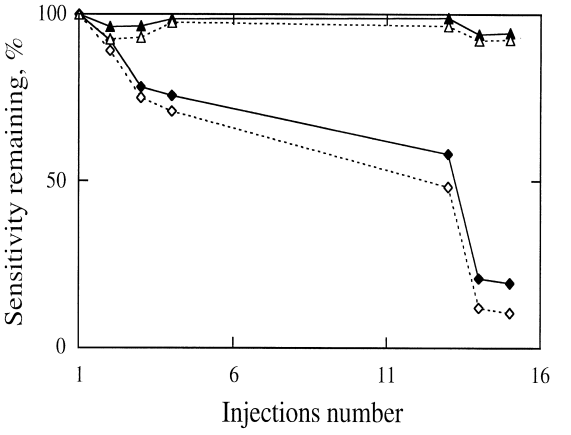

Fig. 3. Decrease of the ED response upon repeated injections of Clari (solid lines) or Roxi (dotted lines) observed when mobile phase with 50% ACN (♦, ◇) or 80% MeOH (▲, △) was used. The first five points refer to injections made within the first working day whereas the two latter points refer to the next working day.

as shown in Fig. 3, probably due to the fact that the electrode cannot partially recover since Roxi eluted after Clari in all eluents used. Consequently, the change of peak area of Clari relative to Roxi within a working day is another indication of electrode deterioration.

3.1.2. Influence of temperature on the ED response and peak shape

Temperature is one of the important factors which may affect the peak area and shape of the macrolides tested in addition to their retention time. For this reason column temperature was the next parameter examined in this work for its influence on the detection quality of Clari and Roxi since a systematic investigation of the retention behavior of these solutes at different temperatures has already been made [13].

As shown in Fig. 4, temperature has a significant effect on ED response and peak shape. Note that in this figure relative peak area means the ratio peak area at a temperature/peak area at room temperature and the relative peak asymmetry at 10% of peak height is the ratio peak asymmetry at a temperature/peak asymmetry at room temperature. In general, it was found that an increase in temperature increased peak area and improved peak shape in all mobile

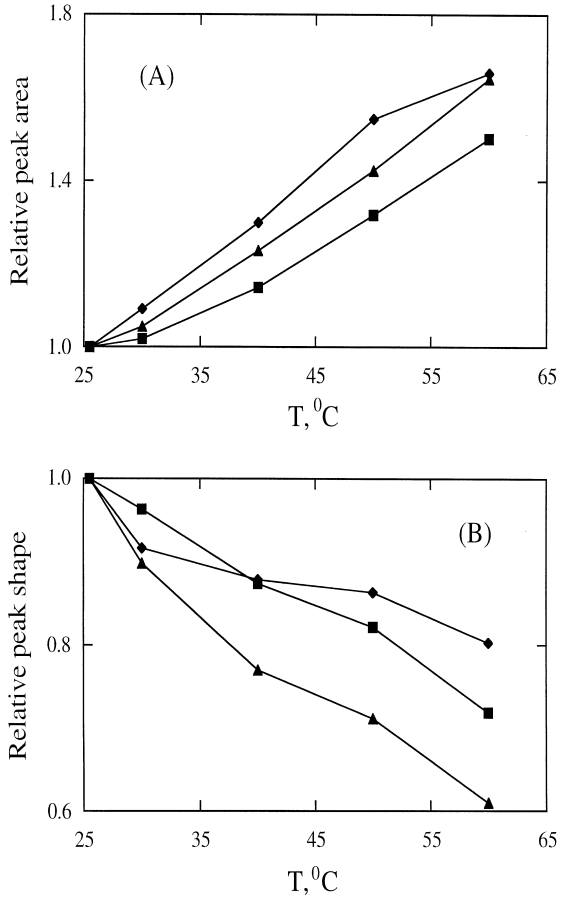

Fig. 4. Effect of column temperature on peak area (A) and peak shape (B) of eluted Clari in mobile phases with different organic modifier: (♦) ACN–iPrOH, (▲) ACN and (■) iPrOH. The exact eluent compositions are indicated in Table 1.

phases used. However, judging from Fig. 4, this effect depended on the eluent system used. For instance, for the same temperature range (25.5–60°C) the increases of the peak area for Clari in the mobile phases of 50% ACN and 28% iPrOH caused by the temperature increase were 65% and 50%, respectively, whereas the corresponding decreases of peak asymmetry were 39% and 28%, respectively.

In addition, higher analysis temperature improved column efficiency for both Clari and Roxi. For example, when increasing temperature from 30 to 60°C an increase of ca. 70% in the theoretical plates was found for both macrolides tested in the mobile

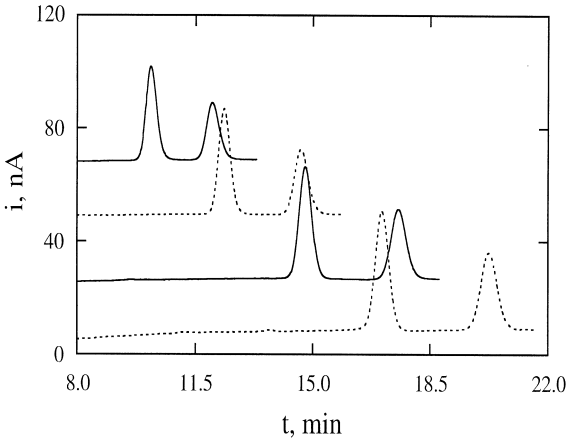

Fig. 5. Chromatograms of 20 μl of a mixture containing 10 μg/ml of Clari (the former peak) and Roxi (the latter peak) using increasing column temperature (30, 40, 50 and 60°C from top to bottom); mobile phase consisting of 55% ACN–iPrOH. See text for other chromatographic conditions.

phase of 55% ACN–iPrOH, where an increase in retention of these compounds was also observed, see Fig. 5.

As concerned the effect of temperature on the passivation of the electrode it was found that the rate of the ED sensitivity decrease was less pronounced with increasing temperature. For example, using ACN as an organic modifier, 50% loss in ED of Clari occurs only after 3 working days. Therefore, the analytical column needs to be maintained at a constant temperature to ensure reproducible responses for both Clari and Roxi.

In general, it was found that an increase in temperature raised ED responses for both solutes, improved peak shape and sensitivity loss but concomitantly increased background current. For these reasons, the analytical column should be thermostatted between 40 and 50°C.

3.2. Linearity and detection limits

Linearity was noted for both macrolides examined at least from the limits of detection to 10 μg/ml with correlation coefficients greater than 0.998 and *y*-intercepts of nearly zero. Standard curves constructed with six different concentrations of Clari and Roxi over the whole range covered the concen-

trations typically found in human plasma after administration of therapeutical doses of these antibiotics. The limit of detection was 0.1 μg/ml (S/N= 3.4 for Clari and 2.5 for Roxi) when injecting 50 μl of a sample at 40°C using mobile phase with 80% MeOH, where the lowest sensitivity was appeared.

Note that detection sensitivity is also dependent on the volume injected. Thus, varying the loop from 20 to 50 μl led to an increase of the ED response by a factor of~2.5 [for example the sensitivity increased from 44.5 to 113 nC/(μg/ml) for Clari] when the above chromatographic conditions were used.

3.3. Application to biological samples

Some of the analytical conditions examined in this issue, particularly column temperature 40°C and mobile phase with 80% MeOH, were applied to the Clari assay in human plasma using Roxi as an internal standard and vice versa.

The recoveries of both Clari and Roxi from plasma samples were estimated by comparing the peak areas after SPE with those obtained by injecting the same amount of an aqueous solution directly into the analytical column. High recoveries of both drugs were found when Oasis cartridges were used and a buffer with pH 10.6 was chosen for the activation of the extraction column as well as for the washing buffer in order to suppress ionization of amino groups of the macrolides and consequently to get strong retardation on the cartridge. The yields of extraction were 93.4±3.8 (n=7) for Clari and 87.7±3.2 (n=7) for Roxi at a concentration 1 μg/ml for both compounds. In addition, no interfering peaks were found in chromatograms from antibiotic-free plasma samples. However, two late peaks with retention times ca. 38 and 43 min, respectively, which delayed the next injection, were found. Fortunately, these interfering background peaks disappeared when only disposable glass tubes were used during the SPE procedure.

In conclusion, the above described SPE procedure seems to be ideally suited for the chromatographic conditions of this investigation and is much more preferable than the liquid–liquid extraction method also tested in this work at alkaline pH with *tert*.-butyl methyl ether as organic solvent.

4. Conclusions

It was demonstrated that the choice of the eluent system and column temperature is crucial in the development of a reliable assay of both Clari and Roxi.

Among the seven different mobile phases examined, the ternary eluent consisting of 75% MeOH–ACN appears to be favored since as a ternary mobile phase usually provides sufficient selectivity to separate most sample mixtures. In addition, the 75% MeOH–ACN mobile phase offers increased sensitivity, improved peak shape and no significant loss of sensitivity due to poisoning of the working electrode.

The column should be thermostatted due to the temperature sensitivity of the ED system. Temperature control at 40–50°C is required to improve peak shape and the signal-to-noise ratio.

A study of the SPE and clean-up of these antibiotics from plasma showed that there were no interfering background peaks present at the retention times of these drugs. High recoveries of both drugs were also achieved.

We believe that our results can also solve analytical problems with other macrolides and/or in different biological fluids with minor modifications.

5. Future directions

In future, it will be of interest to explore the possibility of using a carbon microelectrode as an amperometric sensor for macrolides. Due to the high potential required for oxidation of macrolides and the subsequent high background current, a microelectrode which can be used in a mobile phase without added electrolyte should be the most advantageous.

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