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Determination of Ro 14-4767 (Loceryl™) by LC using automated column switching with ultraviolet and electrochemical detection*

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Abstract: Ro 14-4767, Loceryl™ is a member of a new class of antimycotics, and a number of dosage forms are being developed for its topical application. The active, impurities and its major oxidation products are amenable to ultraviolet detection at wavelengths of approximately 220 nm. As new delivery systems and lower strengths have been developed, it was recognized that a more sensitive detection method may be necessary to support product development. Ro 14-4767, Ro 14-3168 (impurity) and diastereomers are readily oxidized at potentials of 0.8 V (vs Ag/AgCl) at a glassy carbon electrode. Electrochemical detection substantially improved the sensitivity relative to UV detection. However, detection of impurities and oxidation products by either UV or electrochemical detection exhibited interference problems from the cream matrix. Employing column switching eliminated the interference problem from the cream matrix and adding tetrahydrofuran to the mobile phase (acetonitrile and pH 7.0 phosphate buffer) significantly increased the selectivity and sensitivity for both UV and electrochemical detection. The method requires the use of two pumps to continually deliver the mobile phase through both detectors before and after valve switching, preventing the baseline from shifting during a stop in the flow to the electrochemical detector. Quantitative recovery of the active, the potential degradation products, and Ro 14-3168 from a placebo cream using column switching and UV detection has been demonstrated. Utilizing this methodology it is possible to quantitate 0.1% of the formulation label claim for the potential degradation products and Ro 14-3168. Electrochemical detection provides greater selectivity in that only Ro 14-3168, Ro 14-4767 and its diastereomers are detected. However, the sensitivity of detection relative to UV is enhanced. The method is linear in the range of 50 to 150% of the working standard solution for 0.01% and 0.001% active concentration of the cream. The column switching technique can be automated for reproducible assay of a large number of samples.

Keywords: *Oxidization; electrochemical; column switching; selectivity; reproducibility.*

Introduction

Ro 14-4767[4-(3-(*p*-(1,1-dimethyl-propyl)-phenyl)2-methyl-propyl)-2,6-cis-dimethyl-morpholine hydrochloride], Loceryl™ is a cyclic amine member of the morpholine class of compounds [1]. It exhibits a greater antimycotic activity than the better known imidazoles and triazoles which act as ergosterol biosynthesis inhibitors protecting cereal grain against fungal diseases [2]. The improved potency of Loceryl is a result of the morpholine reacting with selective target enzymes. Since the compound exhibits antimycotic activity against a number of dermatophytes, several topical delivery systems have been formulated. Three dosage forms including a solution, nail lacquer and cream were developed for topical use. Analysis of Ro 14-4767 from several dosage forms has been accomplished utilizing high-performance liquid chromatography coupled with UV detection. A partial separation of an impurity (Ro 14-3168) degradation products (Ro 40-1021 and Ro 16-8652) and diastereomers was achieved using column switching [3]. A phosphate buffer at a pH between 7.0 and 7.5 is utilized in the mobile phase to suppress ionization of the amine [4], and to improve both the chromatographic behaviour and detection properties of Ro 14-4767. The resolution was improved by the addition of tetrahydrofuran to the mobile phase.

Ro 14-4767 absorbs weakly in the 200–300 nm ultraviolet spectrum range; the maximum absorption occurring at 220 nm. The quantitative determination of Ro 14-4767 is not always simple due to lack of an inherent

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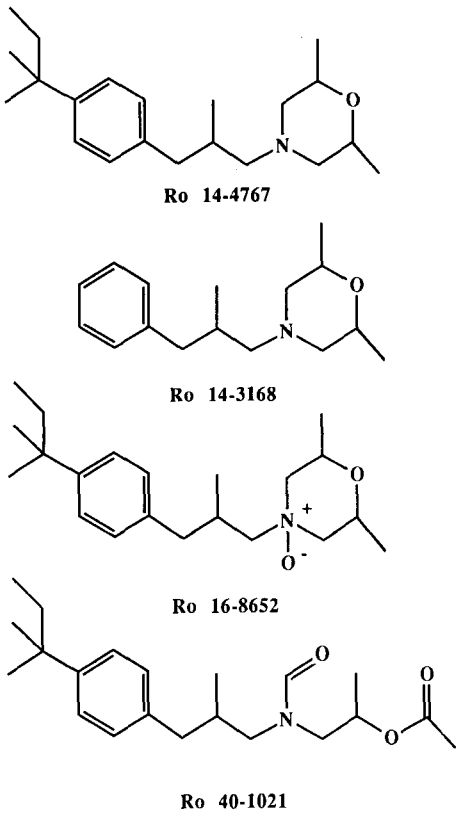

Figure 1
Structures of Ro-14-4767 and related compounds.

chromophore. Since Loceryl contains a morpholine group, the active drug can undergo oxidation when present in the unprotonated form [5]. This oxidation can be monitored with an electrochemical (EC) detector.

In this paper a sensitive and selective method for the analysis of Ro 14-4767 is reported. The method is capable of quantitating the major impurity, diastereomers and degradation products from several dosage forms using LC with column switching, with detection by both UV and on-line EC.

Experimental section

Chemical and reagents

Ro 14-4767, its impurity, diastereomers and degradation products (Fig. 1) were synthesized by Hoffmann La-Roche (Nutley, NJ, USA) or F. Hoffmann-La Roche (Basle, Switzerland). Potassium phosphate and phosphoric acid were all of analytical grade. Acetonitrile, methanol and tetrahydrofuran were HPLC grade (Fisher Scientific). The water was purified by active charcoal, following deionization in an ion-exchange system (Hydro Corp.).

Equipment and chromatographic conditions

The high-performance liquid chromatographic system included a Waters Associate WISP Model 710B injector and two pumps, a Waters Model 590 programmable and a 600E. A Valco 10-port switching valve (Fig. 2) was designed so that selected fractions eluting from column 1 (pre-column) could be transferred to column 2 (analytical column). A 5 cm pre-column (5 micron particle size, 4.6 mm i.d.) and either a Zorbax C_8, C_{18} or endcapped R_xC_8 15-cm long analytical column was used. The phosphate buffer was 0.05 M potassium phosphate dibasic which was adjusted to 7.0–7.5 with phosphoric acid. The mobile phase

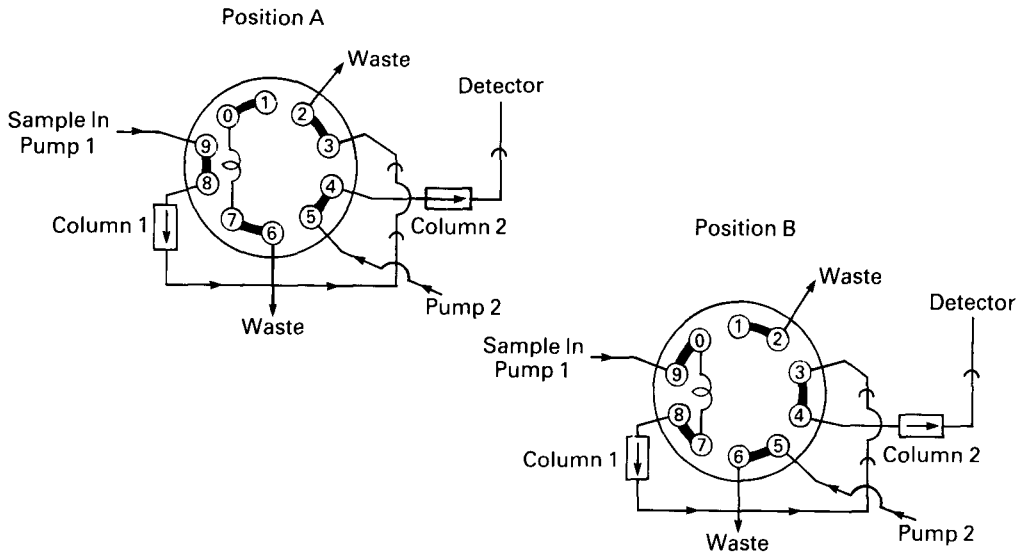

Figure 2
Diagrams of 10-port switching valve assembly.

was a mixture of acetonitrile–tetrahydrofuran–pH 7.0 phosphate buffer (15:15:40, v/v/v) at a 2.5 ml min^{-1} flow rate. Both the pre- and analytical columns were eluted with mobile phase. After injection of the sample, the fractions containing the peaks of interest eluted from pre-column (1) and were transferred to the analytical column (2) where the separation was completed. The specific details of the switching events are summarized in Fig. 3. A *X–Y* recorder (model SE 780 BBC-Metrawatt/Goerz) was used to record the cyclic voltammograms. The applied potential was +0.8 V vs the reference electrode. The peak area integration was performed on a model HP1000 laboratory data system (Hewlett–Packard) and a PE Nelson model 6000 Access*Chrom data system. A Kratos model 783 Spectroflow ultraviolet detector, operated at a 220 nm and an EG&G Princeton Applied Research Model 400 electrochemical detector with a glassy carbon electrode and an Ag/AgCl reference electrode were used. All components of the LC system, including the EC detector, were washed with acetonitrile:distilled water (1:1, v/v) mixture after its use.

Preparation procedure

The nail lacquer and topical creams were diluted with acetonitrile:water (1:1, v/v) and methanol, respectively and 1–20 μg were injected onto the column. The cream samples formulated with Carbonol were diluted using a solvent mixture methanol:water (90:10, v/v), injecting 200–400 μl onto the column. The standard solutions were prepared at the same concentrations as the samples with the same solvent mixture.

Results and Discussion

Chromatography

The quantification of Loceryl impurities and potential degradation products (see Fig. 4) was not possible to monitor from the cream formulation because of the elution of cream excipients. The cream excipients elute during the first few minutes of the assay for Ro 14-4767 in

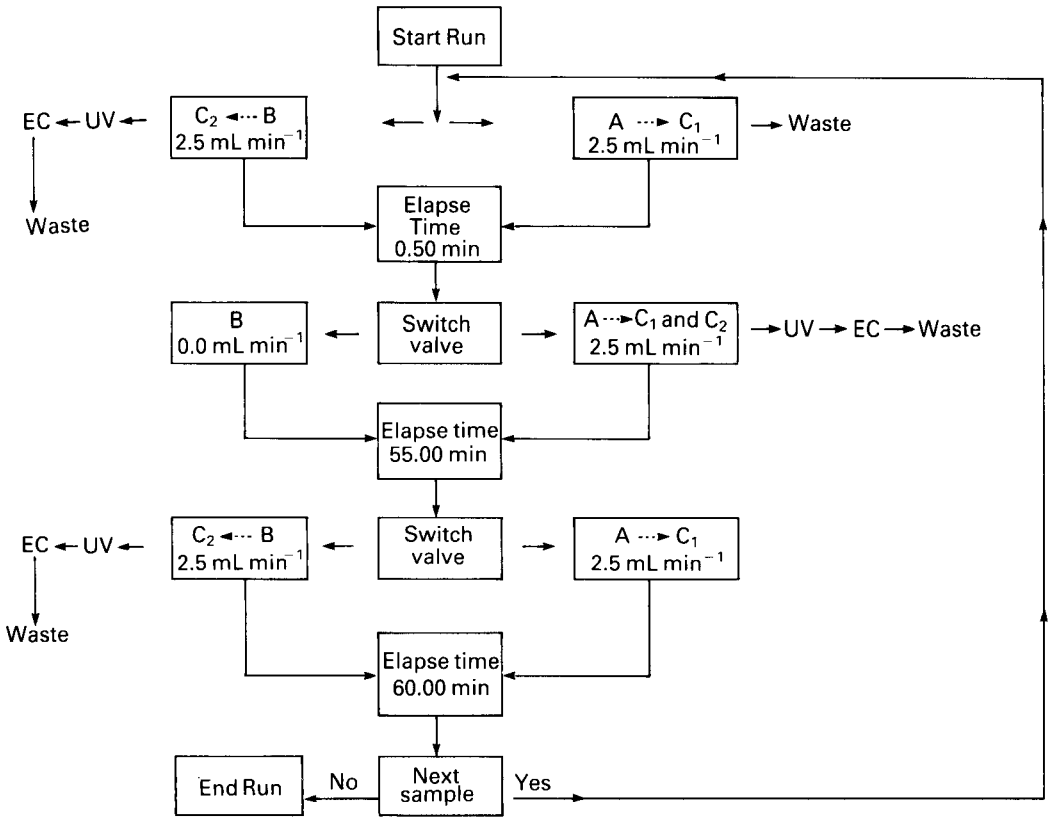

Figure 3
Summary of the switching events. The arrows (--->) inside the boxes indicate the flow of the respective pumps A and B through column 1 (the pre-column) and column 2 (the analytical column). The elapsed times are from the point of injection.

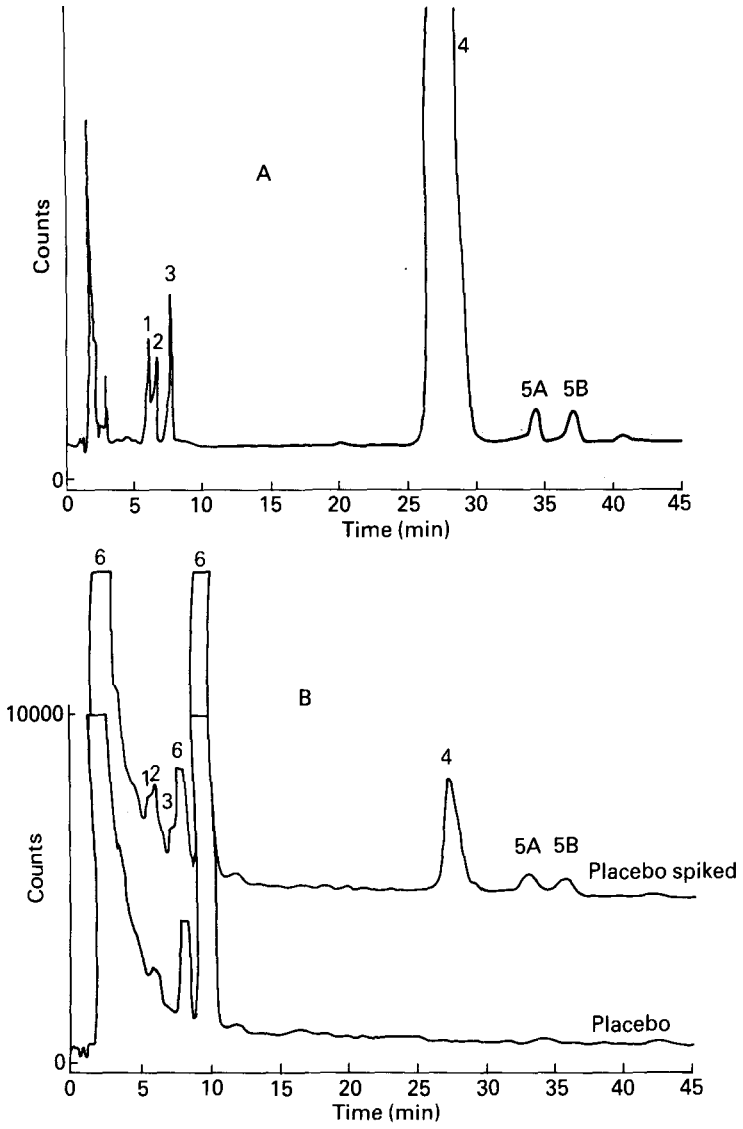

Figure 4
Chromatograms of (A) working standard solution and (B) placebo spiked with working standard solution (no column switching). Pre-column: Novapak C_{18} (5 μm, 15 cm × 4.6 mm, i.d.); analytical column Zorbax C_{18} (5 μm, 15 cm × 4.6 mm, i.d.); injection volume: 400 μl; mobile phase: acetonitrile–phosphate buffer (pH 7.5) (80:35, v/v); flow rate: 2.0 ml min^{-1}; UV detector: 220 nm. Peaks: 1, RO 40-1021 (oxidation product); 2, unknown impurity of Ro 40-1021; 3, Ro 14-3168; 4, Ro 14-4767/004; 5A and 5B. Ro 14-6841 diasteriomers A and B; 6, excipient peak; 7, Ro 16-8652 (oxidation product, see Fig. 6).

the cream formulation, thus it is not possible to monitor the potential oxidative products since they co-elute (Fig. 4). However, by employing column switching to remove excess excipient components a cleaner sample can be delivered onto the analytical column. This separation was achieved by injecting onto a pre-column (Column 1 in Fig. 1; Novapack C_{18}, 5 μm particle size, 4.6 mm i.d., 15 cm long), eluting for 1.30 min, with the eluent that contained fractions of the cream excipient passing to waste. At 1.30 min the switching valve engages and the eluent containing compounds of interest elute from the pre-column to the analytical column (Zorbax C_{18}, 5 μm particle size, 4.6 mm i.d., 15 cm long) where separation is achieved (Fig. 5). The impurity (Ro 14-3168) is well resolved, however, only partial resolution of Ro 40-1021 from an excipient is achieved. No loss of the potential oxidative products nor active drug occurs with the switching technique. The resolution of the oxidative degradation product was completed by adding tetrahydrofuran and increasing the amount of the aqueous component of the mobile phase. The optimum mobile phase mixture was aceto-

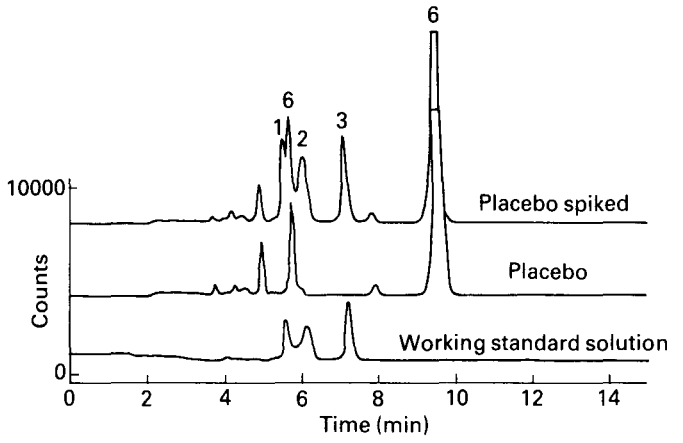

Figure 5
Column-switching separation of working standard solution and placebo spiked with the working standard solution. Conditions and peaks as Fig. 4.

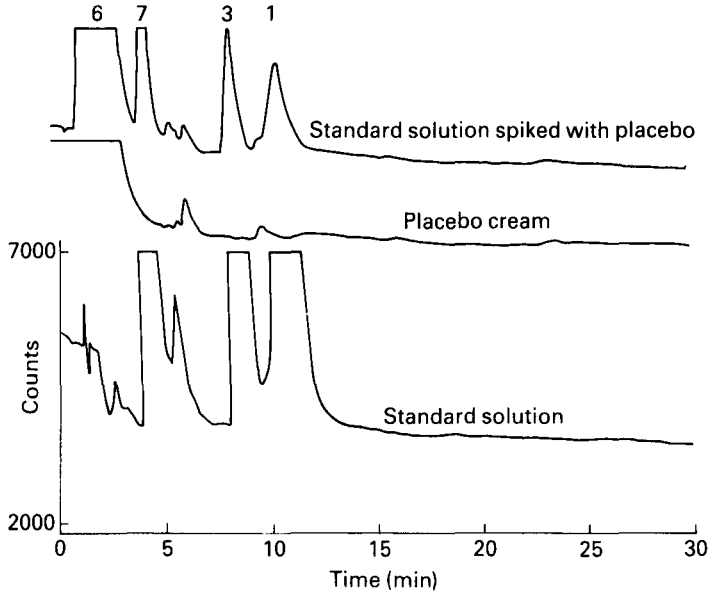

Figure 6
Improved column-switching separation of working standard solution and placebo spiked with the working standard solution. Mobile phase: acetonitrile–tetrahydrofuran–phosphate buffer (pH 7.0) (15:15:40, v/v/v); flow rate: 2.5 ml min^{-1}. Other conditions and peaks as Fig. 4.

nitrile–tetrahydrofuran–pH 7.0 phosphate buffer (15:15:40, v/v/v). The 15 cm pre-column was replaced with a 5 cm pre-column (Novapack C_{18}) to reduce the retention time and back pressure. The flow rate was increased to 2.5 ml min^{-1} and the time of the eluent containing the interfering excipient peak being delivered to waste was shortened to 0.5 min. The achieved separation of the impurity and degradation products including Ro 16-8652 is shown in Fig. 6. The method required the use of two pumps to continuously pump the mobile phase into both detectors before and after the switching valve, preventing the baseline from shifting during a stop in the mobile phase flow.

Electrochemical detection

A cyclic voltammogram of a 3 mM (sample scanned twice) and 0.3 mM Ro 14-4767 solution prepared in deionized purified water are shown in Fig. 7. Full scans were taken from −2000 mV to +2000 mV at a continuous 1.0 ml min^{-1} flow rate. The results of the scans were compared to a blank of deionized purified water. During the scan in the anodic direction an oxidative reaction was observed at a peak

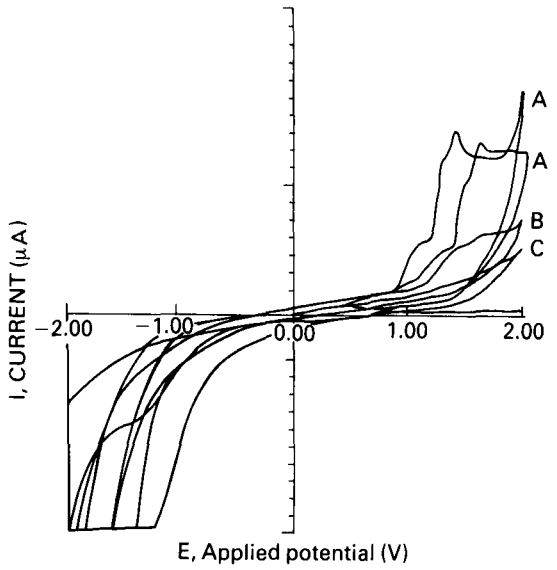

Figure 7
Cyclic voltammogram of Ro 14-4767 in water at a continuous flow rate of 1.0 ml min^{-1}. Current range: 10 μA; scan rate 10 mV sec^{-1}. Key: (A) 3 mM Ro 14-4767; 0.3 mM Ro 14-4767; (C) blank.

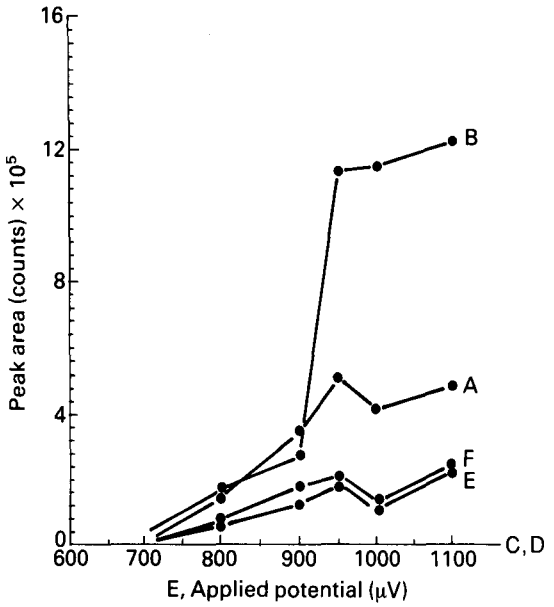

Figure 8
Hydrodynamic voltammograms of Ro 14-4767 and related compounds by flow injection and analysis. Mobile phase: acetonitrile–tetrahydrofuran–phosphate buffer (pH 7.0) (15:15:40, v/v/v); flow rate: 2.5 ml min^{-1}; range: 100 μA full scale; solvent for injection methanol–water (90:10, v/v). Key: (A) Ro 14-4767 (10 μg injected); (B) 14-3168 (13 μg); (C) Ro 40-1021 (10 μg); (D) Ro 16-8652 (30 μg); (E) Ro 14-6841 diasteriomer A; (F) Ro 14-6841 diasteriomer B.

potential at +1.0 V vs a Ag/AgCl reference electrode for the sample at a scan rate of 10 mV sec^{-1}. This oxidation peak was not evident at higher scan rates.

The hydrodynamic voltammograms for Ro 14-4767, its impurity and diastereomers prepared in methanol–water (90:10, v/v), working in mobile phase under flow injection conditions, exhibited no electrode response below +0.7 V and a maximum response at +0.95 V. The mobile phase consists of a mixture of acetonitrile–tetrahydrofuran–pH 7.0 phosphate buffer (15:15:40, v/v), see Fig. 8. Removing the tetrahydrofuran decreased the minimum response to +0.6 V and increased the maximum response to about +1.0 V for Ro 14-4767. No response was observed for the degradation products Ro 40-1021 and Ro 16-8652. Setting the applied potential at +1.0 V resulted in an 86% loss in area response after successive injections. This is because a passivating film formed on the electrode. This film deters the mass transfer of Ro 14-4767, its impurity and diastereomers to the electrode surface. Area response, however, was restored with vigorous polishing of the electrode using an alumina slurry on a microcloth followed by sonication for a few minutes to remove any of the alumina grit. Setting the applied potential at +0.8 V the reproducibility of the injections improved dramatically for Ro 14-4767 as shown in the precision section (Table 1).

Chromatograms of Ro 14-4767 formulated in a nail lacquer (Fig. 9A–D) and topical cream (Fig. 10) were obtained by coupling the electrochemical detector in sequence with an UV detector. Using the Zorbax C_8 (5 μm particle size, 4.6 mm i.d. × 25 cm long) column with a mobile phase of acetonitrile–pH 7.5 phosphate buffer (80:35, v/v) and a flow rate of 3.0 ml min^{-1} the active compounds eluted at 19 min for both detectors used. EC detection is shown to be more sensitive. The EC chromatograms at 10–15% full scale show two excipient peaks eluting before 7 min and a diastereomer peak at a retention time of 28 min. Setting the UV chromatogram at 0.5% full scale these peaks were not evident. Topical creams that were formulated with propyl gallate as an antioxidant were observed to contain a component which interfers with the quanitification of degradation products and impurities. This problem was observed for both UV as well as EC since propyl gallate is also electroactive (see Fig. 11A). As one can see from the chromatogram of the cream

Table 1
System precision results from a Ro 14-4767 reference standard separate solution

	Area response (mV-sec)*			Area response (Counts)† Using column switching (UV detection)‡			
	UV	Electrochemical detection					
	220 nm	Microamps	Nanoamps	Topical cream concentration (%)			
Injection number	22.0 mcg	21.3 mcg	213 ng	0.25	0.125	0.01	0.001§
1	9966.500	420.989	20386.109	63071298	63001140	6175628	1236504
2	9978.650	411.823	20535.593	63062360	62703410	6198646	1233511
3	9954.162	412.807	20296.968	61340692	63329776	6160185	1210951
4	9994.623	400.438	20742.281	63355753	63145942	6139239	1192973
5	10035.503	405.306	20381.218	62693084	62598443	6113669	1217684
6	10019.451	405.905	21188.906	61529537	61747327	6147508	1189535
x̄ (mean)	9991.482	409.547	20588.513	62508787	62754340	6155813	1213526
SD	31.342	7.22	333.423	859948	563269	29551	19745
%RSD	0.3	1.80	1.62	1.4	0.9	0.5	1.6

*Peak area integration by a Model HP 1000 Laboratory data system (Hewlett–Packard).
†Peak area integration by a PE Nelson Model 6000 Access *Chrom GC–LC data system.
‡Computer output 1 V full scale.
§Computer output 10 mV full scale.

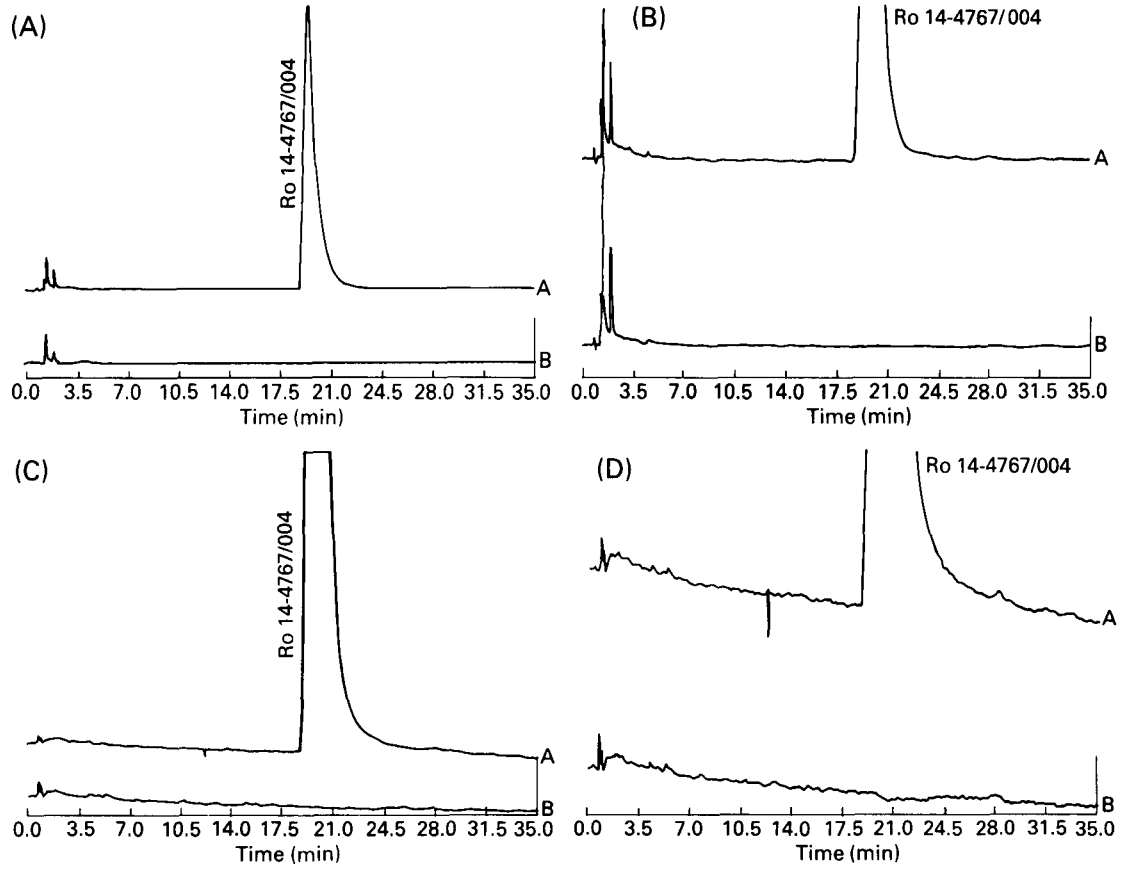

Figure 9
Chromatograms of Ro 14-4767 in a 5% nail lacquer formation by UV and EC detection. (A) and (B) UV detection at 100 and 0.5% full scale, respectively; (C) and (D) EC detection at 100 and 10% full scale, respectively. The upper traces represent the formulations and the lower traces represent the blanks. Flow rate 3.0 ml min^{-1}. EC applied voltage: 0.8 V. Other conditions as Fig. 6.

extract, the sensitivity of Ro 14-3168 (impurity) decreases as it elutes off the shoulder of the cream excipient. With the use of column switching the sensitivity of the impurity peak increases as fractions of the cream excipient are removed from the chromatogram. This is also evident in Fig. 11B. The UV was on-line to detect the degradation products that were not electroactive and could not be observed with the EC detector.

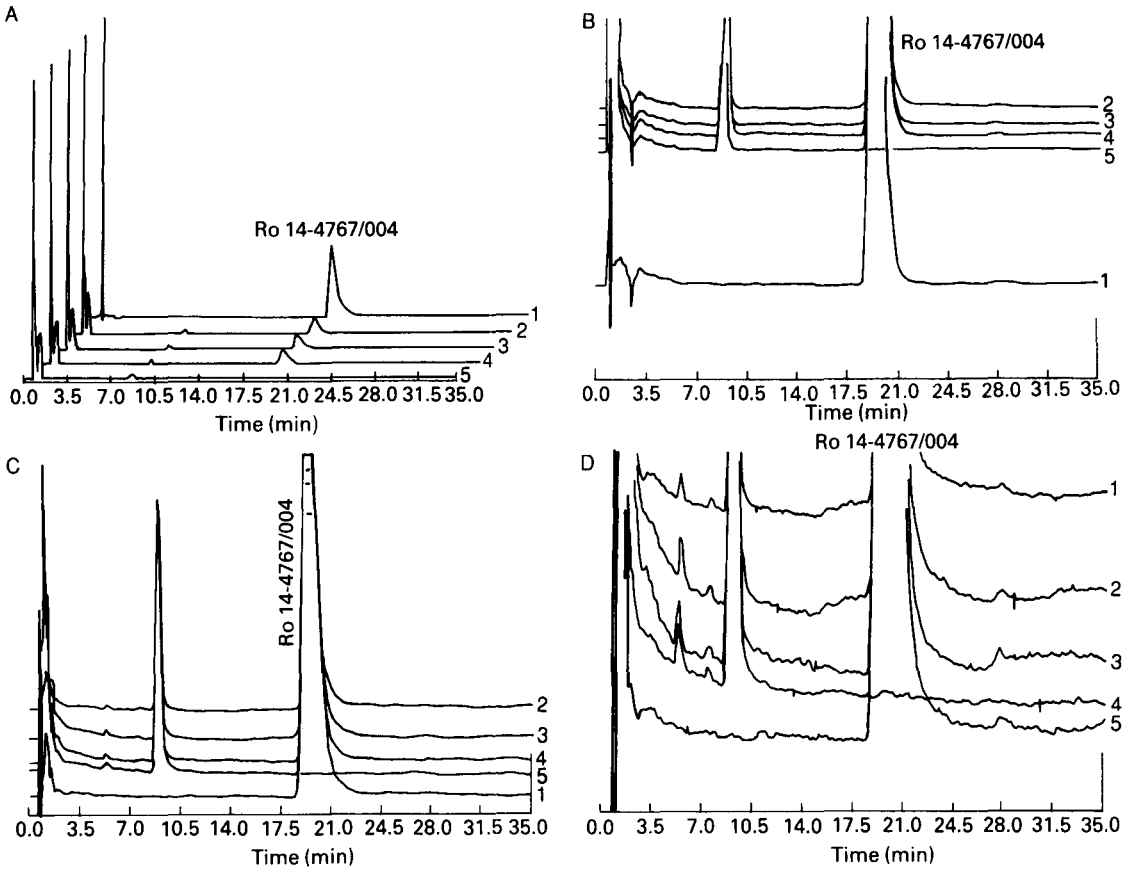

Figure 10
Chromatograms of Ro 14-4767 in a 0.25% topical cream formulation following storage at various temperatures for 1 month by UV and EC detection. (A) and (B) UV chromatograms at 100 and 0.5% full scale, respectively; (C) and (D) EC chromatograms at 100 and 15% full scale, respectively. Conditions as Fig. 9. Key: (1) control; (2) 45°C; (3) 37°C; (4) 5°C; (5) placebo.

Validation

System precision. The system precision is obtained from six consecutive injections of Ro 14-4767 reference standard solution. Reproducibility was studied with an EC detector set at both the microampere and nanoampere current ranges at an applied potential of +0.80 V. Data for EC detection was compared with that from UV detection. Results utilizing the nanoampere current range were obtained for a 100-fold dilution of this standard solution. A separate study included the use of column switching with UV detection. This study was based on the four working concentrations for topical cream formulated at various concentrations. For a 0.25 and 0.125% topical cream preparation of the reference standard solution was the same, however, for the 0.01 and 0.001% topical cream the solution is diluted 10 and 50-fold, respectively. The system precision obtained from six replicate injections of each standard solution is between 0.3 and 1.8% relative standard deviation (Table 1).

Linearity. The LC with EC detection method for RO 14-4767 was shown to be linear between 50 and 150% of the expected working concentration range. Setting the EC detector at the microampere current range the response is linear from 10 to 30 mcg of drug injected onto the column (r = 0.9955) and linear with a 100-fold dilution of these solutions at the nanoampere current range setting (r = 0.9922). The use of column switching, with two 15 cm columns in series and by UV detection, the method is linear (r = 0.9961–0.9983) at the range of 50–150% of the standard solution for lower drug concentration strengths (0.01 and 0.001% cream). However, linearity is observed only at the range of 50–110% (r = 0.9871) at higher concentration strengths (0.125 and 0.25% cream) of active drug due to band broadening.

Recovery. Recovery of added drug was demonstrated (Table 2a and b) by preparing six samples of Ro 14-4767 nail lacquer ranging

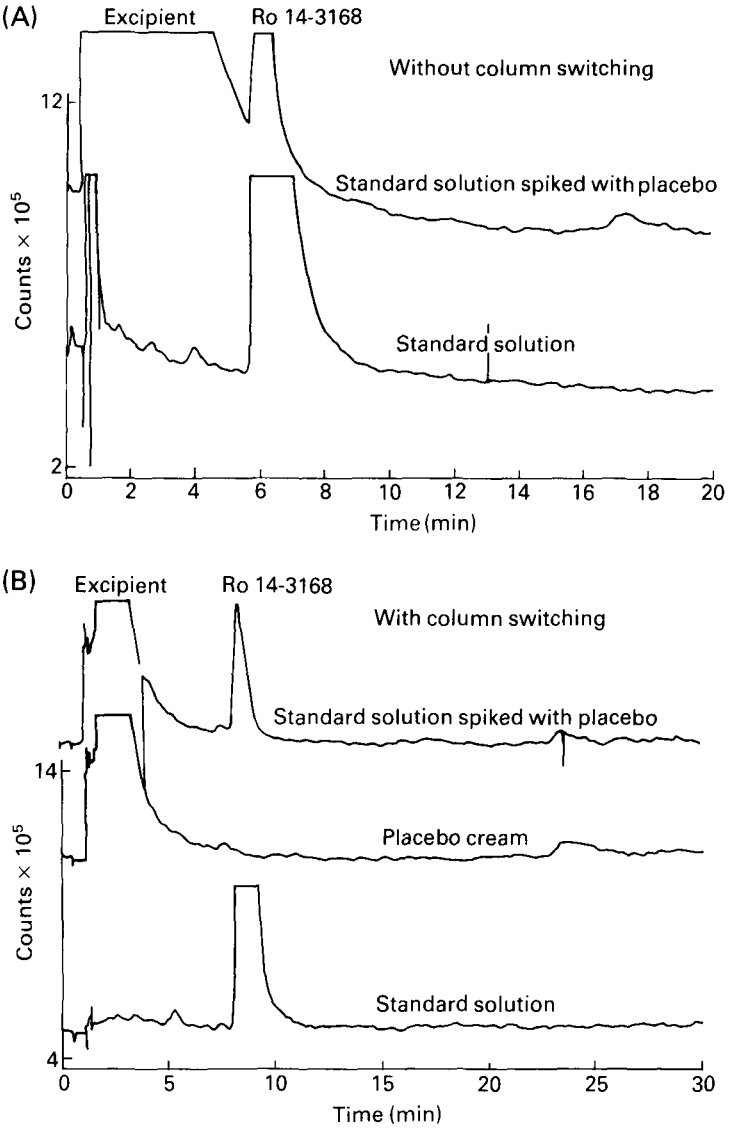

Figure 11
Effect of column switching on the separation of Ro 16-4767 from a 0.25% topical cream formulation using EC detection. (A) Without column switching, (B) with column switching. Conditions as Fig. 6.

from 50 to 150% of the concentration used in the assay. Column switching was not used for the analysis of the nail lacquer. For the topical cream, six samples of the placebo were spiked with Ro 14-4767/004 at a concentration used in the assay were analysed with the use of column switching. The range of recovery obtained by both methods is 98 to 106% of label claim with a relative standard deviation of less than 1.6%. Detection was determined by UV at 220 nm.

Sensitivity. The use of an electrochemical detection provides increased sensitivity of detection for Ro 14-4767. The area response of the working standard concentration increased 200 times vs UV detection when the current range on the EC detector was set at the nanoampere range at an applied potential of +0.80 V (see Table 1). The hydrodynamic voltammogram shows that increasing the applied potential should maximize analyte response. However, oxidation of the eluent with the concomitant electrode and the formation of a passavating film on the surface of the electrode at applied potentials greater than +0.8 V leads to a higher background current. Baseline noise and drift are proportional to background current and so higher applied potentials may decrease the signal to noise ratio. The limit of the UV detection of Ro 14-4767 (20 ng injected onto the column) was

Table 2a
Recovery of Ro 14-4767: nail lacquer

Theory (mg ml^{-1}) of Ro 14-4767	Observed (mg ml^{-1}) of Ro 14-4767	% Recovery
77.545	81.127	105
63.145	65.255	103
57.310	59.866	105
51.835	53.413	103
44.120	45.085	102
38.615	40.095	104
24.055	24.547	102
	$\bar{x}$ (Mean)	103
	SD	0.5
	%RSD	0.5

Table 2b
Recovery of Ro 14-4767: topical cream

	Recovery of Ro 14-4767/004 from topical cream (using column switching)							
	0.25% Cream*		0.125% Cream†		0.01% Cream‡		0.001% Cream§	
Sample number	Observed (mg)	% Recovery	Observed (mg)	% Recovery	Observed (mg)	% Recovery	Observed (mg)	% Recovery
1	2.523	98	1.260	98	135.664	106	10.274	100
2	2.523	98	1.254	98	136.170	106	10.250	100
3	2.454	96	1.267	99	135.325	106	10.062	98
4	2.534	99	1.263	99	134.865	105	9.913	97
5	2.508	98	1.252	98	134.303	105	10.118	99
6	2.461	96	1.235	96	135.046	105	9.884	96
$\bar{x}$ (Mean)		98		98		106		98
SD		1.2		1.1		0.6		1.6
%RSD		1.3		1.1		0.5		1.6

*Theoretical value is 2.563 mg.
†Theoretical value is 1.282 mg.
‡Theoretical value is 128.2 mcg.
§Theoretical value is 10.3 mcg.

estimated by determining the concentration equivalent to approximately three times the detector noise. The area response for EC detection is 50 times greater. The peak height response of Ro 14-4767 with the UV detector is about 3 mV detector output, whereas with the EC detector the output is greater than 90 mV.

Conclusions

System precision, recovery of active drug from various formulations as well as the linearity of response have been demonstrated for an HPLC separation of Ro 14-4767 and its major impurity, degradation products and diastereomers utilizing column switching and on-line UV and EC detectors. Utilizing column switching it is possible to quantitate 0.1% of the formulation label claim for the potential degradation products and Ro 14-3168. Electrochemical detection provides greater selectivity with only Ro 14-3168, Ro 14-4767 and its diasteromers detected. The sensitivity of detection relative to UV is enhanced as much as 200-fold if the currents are set at the nanoampere range with an applied potential of +0.8 V. Both methods are linear in the range of 50 to 150% of the working standard solution for 0.25 to 0.001% range at active concentration of the cream. However, using column switching band broadening is observed beyond 110% linear range for the higher concentrations (0.125 and 0.25% cream) of active drug. The column switching technique can be automated for reproducibility assay of a large number of samples.

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