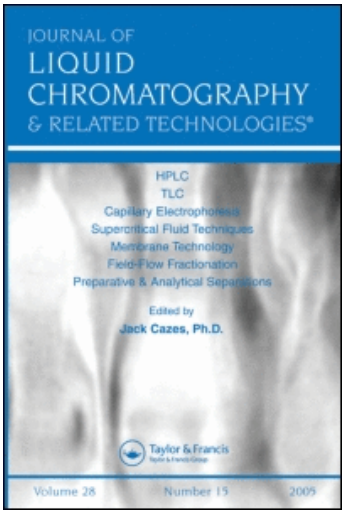

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Determination of Hydrocortisone and Associated Compounds in Pharmaceutical Preparations by Micellar Electrokinetic Chromatography

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ABSTRACT

A method for quantifying hydrocortisone (HYD), hydrocortisone acetate (HYDA), oxytetracycline (OXY), Zn-bacitracin (BAC), and polymyxin B (PLX) in pharmaceutical products by micellar electrokinetic chromatography (MEKC) is described. The separation was carried out at 25°C and 25 kV, using a 15 mM phosphate–15 mM borate buffer (pH = 8.2), 60 mM sodium dodecyl sulfate (SDS), and 10% methanol–water (v/v) as a background electrolyte. Under these conditions, the analysis takes about 23 min. The method has been applied for quantifying these compounds in four different commercial pharmaceutical products, and the method gave good results when compared with a reference spectrophotometric method.

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Key Words: Hydrocortisone; Oxytetracycline; Zn-bacitracin; Polymyxin B; Micellar electrokinetic chromatography.

INTRODUCTION

Corticosteroids have been widely used as anti-inflammatory drugs in medicine. Nowadays, commercial pharmaceuticals contain corticosteroids in conjunction with anti-bacterials, because corticosteroids do not solve the fundamental reason of the disease, and as a result can cause masking of the real disease (for example, a infection).

These compounds are used very efficiently for a wide range of ocular, allergic, and cutaneous inflammatory diseases, so there are many formulations [hydrocortisone acetate–oxytetracycline–polymyxin B (HYDA–OXY–PLX), hydrocortisone acetate–bacitracin (HYDA–BAC), or hydrocortisone–oxytetracycline (HYD–OXY)], and concentrations of corticosteroids in variable doses for local administration.

Hydrocortisone is a human glucocorticosteroid, which is usually associated with antibiotics.[1,2] The most important antibiotics in local administration are OXY (an important tetracycline characterized by a broad-spectrum activity against pathogenic microorganisms),[3] BAC (a polypeptide antibacterial derived form of *Bacillus licheniformis* and *Bacillus subtili*, having some advantage against other antibiotics because it does not produce hypersensitivity in ointments),[4] and PLX (a basic, macro cyclic peptide antibiotic that is derived from *Bacillus polymyxa* and used against many Gram-negative bacterial infections in local administration).[2]

Hydrocortisone is mainly determined by spectrophotometry,[5,6] by HPLC in reverse phase in plasma and pharmaceuticals, UV detection generally at 254 nm in association with other compounds and antibiotics,[7–10] and by selective micellar capillary electrophoresis micro assay developed for the determination of HYD in urine.[11]

The spectrophotometric methods to determine OXY were based on direct methods,[12] complexation reactions,[13,14] and derivative spectrophotometry.[15–17] The most widely used technique for simultaneous determination is HPLC.[18,19] Oxytetracycline and its degradation products can be determined in synthetic and biological samples by capillary zone electrophoresis (CZE)[20,21] and micellar electrokinetic chromatography (MEKC).[22]

The most widely used technique for simultaneous determination of BAC A and the other bacitracins is HPLC with derivatization[23] in reverse phase chromatography, and by TLC in association with PLX and other antibiotics[24] and nonaqueous capillary electrophoresis–mass spectrometry (NACE–MS),[25] and by MEKC.[26]

Polymyxin B can be determined spectrophotometrically by derivative and multivariate calibration techniques in pharmaceuticals and in synthetic mixtures, simultaneously, with dexamethasone and trimethoprim.[27,28] A methodology is described for the detection of PLX by reverse-phase HPLC using a linear gradient.[29] It is possible to detect PLX by CZE[30] and quantification of PLX by MEKC, in association with dexamethasone and trimethoprim, in different pharmaceutical formulations.[31]

In this work, the separation and quantification of hydrocortisone, hydrocortisone acetate, and these associated compounds were studied. No references were found for the quantification of BAC, PLX, and the simultaneous determination and quantification of these associated compounds by capillary electrophoresis. Our group has been doing research, for a long time, into the possibilities offered by CE (rapid set-up of instrumentation, versatility and low cost) for the determination of corticosteroids and their more important associated compounds in commercial pharmaceuticals.[31,32] As a result, regarding the routine analysis of these drugs, this paper presents a new, accurate, and easy MEKC method for the determination of the mentioned mixtures. The structures of these compounds are given in Fig. 1.

EXPERIMENTAL

Apparatus

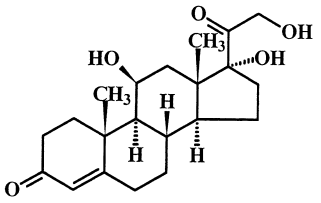
A Beckman P/ACE 5510 (Fullerton, CA) capillary electrophoresis system equipped with a diode-array detector was used. The system was controlled by a Dell Dimension™ P133V with P/ACE Station Software. Separation was carried out on a 57 cm (50 cm to the detector) × 75 μm i.d. fused silica capillary housed in a cartridge with a detector window 800 × 100 mm.

A Crison (Barcelona, Spain) MicropH 2002 pH meter was used for the pH measurements.

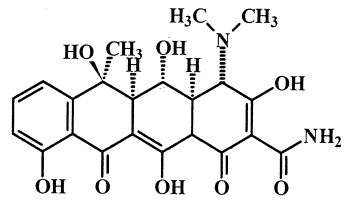
A Beckman (Fullerton, CA) DU-70 spectrophotometer equipped with 1.0 cm quartz cells and connected to an IBM-PS 2Model 30 computer, fitted with Beckman Data Leader software, was used.

Reagents and Solutions

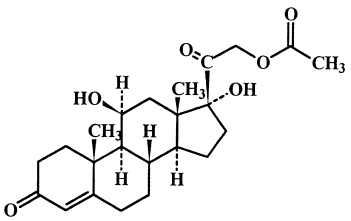
All solvents and reagents were of analytical grade unless indicated otherwise. Solutions were prepared with deionised water (Milli-Q quality). HYD, HYDA, OXY hydrochloride, BAC, and PLX were obtained from Sigma (Germany).



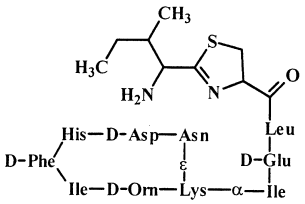
HYDROCORTISONE



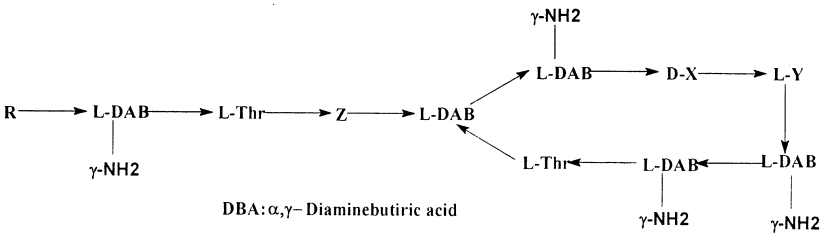
OXYTETRACYCLINE



HYDROCORTISONE 21-ACETATE



BACITRACIN A



Polymyxin B1:R=(+)-6-methiloctanoil;X:phenilalanine;Y:leucine;Z:L-DBA
Polymyxin B2:R=6-metilheptanoil;X:leucine;Y:treonine;Z:D-serine.

POLYMYXIN B

Figure 1. Chemical structures of the mixture compounds.

Stock solutions (200 mg/L) of HYD, HYDA, and BAC were prepared in methanol–water (50 : 50) and OXY and PLX stock solutions were prepared in water.

Buffer solutions were prepared by dissolving the adequate quantity of NaH_2PO_4 and $Na_2B_4O_7$ in deionized water and then adjusting with HCl or

NaOH to the required pH. All these reagents were from Panreac (Barcelona, Spain).

Operating Conditions

Separations were performed using 4 mL glass vials. The rinse step was carried out using vials, in order to keep the level of buffer constant in the anodic separation vial. The set of separation vials was changed after each batch was run (maximum 4 separations). The capillary was conditioned prior to its first use by flushing, first with 0.1 M NaOH for 20 min, then with water for 10 min. In the optimum method, the capillary was washed with 0.1 M NaOH under high pressure for 2 min, then filled for 2 min with the separation buffer, followed by a 6 s hydrodynamic sample injection. The separation was performed at 25 kV for 24 min at 25°C under the selected conditions, the current was 63.0 μA.

The data generated from the first two injections in a sequence were not used as required by system equilibration. Injections of the solutions among the standard were performed and peak area was used for the quantification.

RESULTS AND DISCUSSION

Preliminary Studies

To optimize separations, a preliminary study was carried out using a solution containing 16, 16, 32, 32, 64 mg/L of HYD, HYDA, OXY, BAC, and PLX, respectively. A 20 mM phosphate buffer (pH 7) with 30 mM SDS as electrolyte solution was used; temperature and voltage were 25°C and 25 kV, respectively.

Influence of the pH on the Separation

Separations have been carried out at different pH values (6, 7, 8, 9, 10, 11) with and without SDS. The results demonstrated the separation is better when the pH is 8 and when the background electrolyte contains SDS as surfactant. By these separations it could be proven that OXY, BAC, and PLX are ionic forms under the described conditions, so they appear away from the electro-osmotic flow (EOF) when the surfactant was not added to the electrolyte, while HYD and HYDA eluate with the EOF in all those cases.

Sodium dodecyl sulfate was selected as a micellar additive in the electrolyte as it is the most common surfactant used in MEKC. A phosphate–borate (1 : 1) buffer at pH 8.2 was chosen in our study due to the high buffer ability of the borate (pK_a 9.2) and the high buffer ability of the phosphate (pK_a 7.5).

Influence of the Organic Modifier

Preliminary experiments suggested addition of some kind of organic modifiers, because some peaks (PLX and BAC) were not well resolved and showed shoulders. The experiments were performed using 20 mM phosphate–borate buffer, pH 8.2 containing 30 mM SDS as electrolyte. Methanol and acetonitrile were tested in concentrations from 3 to 12%. The presence of 10% of methanol in the electrolyte showed well resolved peaks, and shoulders disappeared.

Influence of Phosphate–Borate (1 : 1) Buffer Concentration

The phosphate–borate buffer molarity was varied from 10 to 50 mM using the experimental conditions mentioned above, and its influence upon the migration time was studied. A 30 mM (15 mM phosphate–15 mM borate) concentration was considered suitable for its good resolution and peak slope, whereas higher concentrations resulted in peak broadening.

Influence of Sodium Dodecyl Sulfate

The influence of SDS in the electrolyte on the migration time is given in Fig. 2. The results demonstrate that the SDS concentration dramatically affects the mobility of the HYD, HYDA, PLX, and BAC [Fig. 2(a)]. A concentration of 60 mM was selected for the experiment to give the best resolution [Fig. 2(b)]. The current generated was 64.5 μA and the run time was about 18 min.

Influence of Running Voltage and Temperature

The maximum voltage can be determined from the graph of observed current vs. applied voltage (Ohm's law plot). Running voltages in the range 5–30 kV were tested by using the above experimental conditions. This graph was linear up to 25 kV. As expected, decreasing migration times were obtained with increasing applied voltages. Higher voltages give shorter analysis times, higher efficiencies, and, on the other hand, higher currents and increasing Joule heating. But, if the negative effects are controlled, a potential of 25 kV can be selected as optimum.

A temperature lower than 20°C was not considered because the surfactant has enough solubility to form micelles only at temperatures above the Kraft point (16°C for SDS); and temperature regulation with the instrument is efficient only until 4°C below room temperature. We investigated the effect of temperature on the separation between 20 and 35°C by employing the selected condition (30 mM borate–phosphate (50 : 50) buffer pH 8.2; 60 mM SDS; 25 kV).

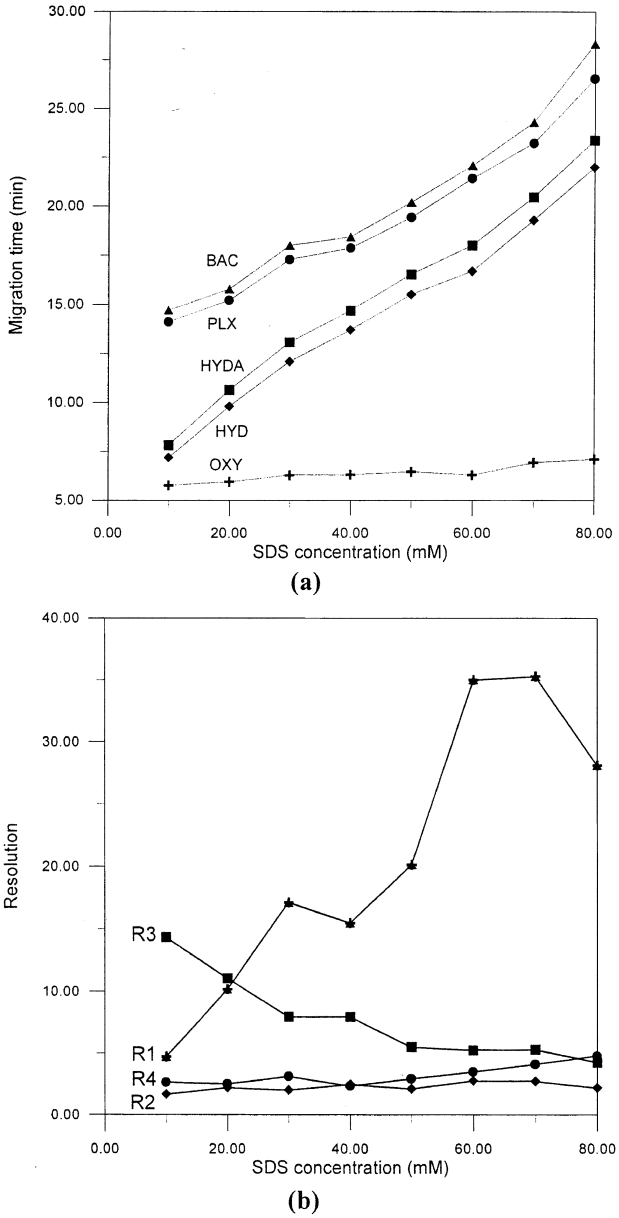


Figure 2. Influence of the SDS concentration on the migration time (a), and on the resolution (b).

For temperature higher than 35°C, contribution of Joule heating and temperature gradient become more pronounced, giving band broadening. 25°C was selected as a compromise between resolution, run time, current intensity, and acceptable level of base-line-noise.

Optimization of Rinsing and Washing Steps

A washing step of 2 min with 0.1 M sodium hydroxide, followed by a 2 min buffer washing, was adequate to restore the capillary wall surface and re-equilibrate the capillary between sample injections.

Selected Conditions

From the studies carried out before, we suggest that the procedure summarized below is convenient to separate the mixture properly (Table 1).

The electropherogram obtained in the separation under selected conditions is presented in Fig. 3. It is remarkable that all peaks have good relations in a run time of 24 min.

Performance Evaluation

Limits of Detection and Quantification

Limits of detection and quantification (LOD and LOQ, respectively) were estimated in accordance to the base line noise method. The base line noise was evaluated by recording the detector response over a period of 10 times the peak width. The LOD was obtained as the sample concentration that causes a peak with a height three-fold the base line noise level,[33] and the LOQ was calculated as ten fold the base line noise level. Thus, LODs and LOQs are shown in Table 2 for each compound.

Table 1. Optimized conditions for the separation.

Capillary	Fused silica (57 cm length × 75 μm inner diameter)
Electrolyte	15 mM phosphate–15 mM borate buffer pH = 8.2; 60 mM SDS and 10% methanol
Temperature	25°C
Voltage	25 kV
Detector	Diode array
Window	800 × 100 μm

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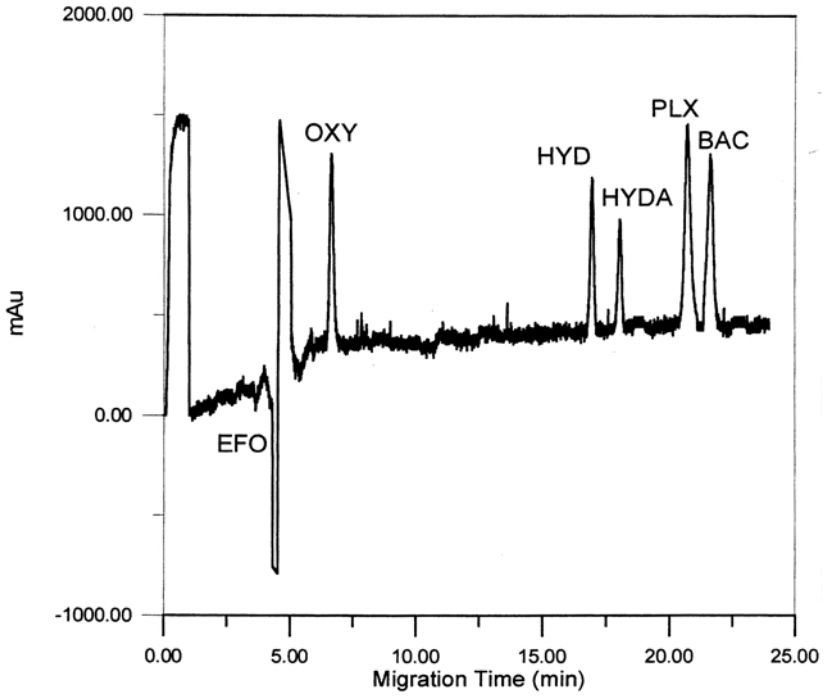

Figure 3. Electropherogram of a sample containing 32, 16, 16, 64, and 32 mg/L of OXY, HYD, HYDA, PLX, and BAC obtained under the optimized conditions at 215 nm.

Linearity Range and Calibration Curves

The linearity of the assay was checked by injecting standard solutions of each drug in the range from 4 to 80 mg/L. In all cases, the separation was carried out by using the optimized electrophoretic procedure. The calibration curves were obtained for each component by plotting the correct area, measured at the maximum absorption wavelength, 267, 245, 245, 205, 205 nm for OXY, HYD, HYDA, PLX, and BAC, respectively, vs. their concentrations.

A good linear relationship was obtained between concentration and corrected area for each component. In Table 2, the slopes, intercepts, r^2, and linearity ranges for the calibration curves are presented. In all cases, the intercepts were estimated as negligible by using the Student's *t*-test ($\alpha = 0.05$).

The middle point of the calibration curves were selected to study the influence of the injection time on the corrected area, from 2 to 10 s; resulting in similar linearity range conditions that were already provided.

Table 2. Limits of detections and LOQs.

	OXY	HYD	HYDA	PLX	BAC
LOD (mg/L)	1.9	0.5	0.4	5.7	2.6
LOQ (mg/L)	6.5	1.6	1.3	19.0	8.5
Intercepts (CAU[a])	16 ± 35	44 ± 60	65 ± 73	-505 ± 77	-228 ± 92
Slope (CAU × L/mg)	64.3 ± 0.8	84.7 ± 1.8	81.1 ± 3.1	81.0 ± 1.6	75.0 ± 4.8
r^2	0.9985	0.9964	0.9913	0.9965	0.9901
Linear range (mg/L)	7.5–75.5	2.5–60.8	2.5–40.0	20.0–80.9	8.6–28.4

Note: Linear regression calibration curves.
[a]Correct area unit.

Repeatability and Reproducibility

Repeatability was assessed under the previously selected conditions by means of 12 replicates of a solution containing 40 mg/L OXY, 24 mg/L HYD and HYDA, 60 mg/L PLX, and 16 mg/L BAC. Reproducibility was evaluated over 2 days by performing 12 replicates each day.

The results showed that the repeatability for every component in each day is satisfactory. In terms of reproducibility, the comparison of averages with the Snedecor test did not provide any significant difference between both days series, for $\alpha = 0.05$ ($n = 12$).[34,35]

Peak Purity

Peak purity was obtained for all compounds by overlapping the spectra captured at the apex, upslope, and down slope. No differences were noted for all components.

Applications

The present method was tested to determine the mentioned compounds in pharmaceutical preparations. The Spanish pharmaceutical industry has, at the present, different commercial formulations containing OXY, HYD, HYDA, PLX, and BAC.

Dermo-Hubber is an ointment with HYDA and BAC from the enterprise Teofarma Ibérica S.A. Terra-Cortril, is an ointment with HYD and OXY from the enterprise Teofarma Ibérica S.A. Terra-Cortril, ótico oftálmica is an ointment with HYDA, OXY, and PLX from the enterprise Teofarma Ibérica

S.A. Terramicina, Tópica, is an ointment with OXY and PLX from the enterprise FarmaSierra S.A.

An amount of each ointment was weighed accurately into an extraction glass. A sequential extraction is made to extract all the compounds with a total volume of 100 mL. Different volumes of 20 mL are shaken and then are subjected to an ultrasonic bath for 15 min, to complete 100 mL. This total volume of the extraction was filtered and different known aliquots were placed in a 25 mL calibrated flak, adding, also, methanol (final solution contained 20% methanol) and deionized water.

In the analysis of the commercials, the found amounts and recoveries were achieved by comparison with standard solutions containing the same concentrations expected for commercials, according to their claimed levels. The standard solutions were prepared from the stock solutions after convenient dilutions.

A multivariate calibration method was developed to confirm the results obtained in MEKC. The same compounds were determined by PLS-1. The results, presented in Table 3, show agreement between the claimed and found values.

In Fig. 4 we can see the electropherogram of a sample of Terra-Cortril ótico oftálmica under the optimised conditions at 215 nm.

CONCLUSION

The results show that MEKC is a valuable technique for the determination of HYD and HYDA Acetate and their associated compounds. Assay and

Table 3. Application results.

		MEKC		PLS-1	
Commercial	Claimed (mg/L)	Found (mg/L)	Recovery (%)	Found (mg/L)	Recovery (%)
Dermo-Hubber	HYDA 44.3	42.2 ± 0.7	95.4	42.8 ± 0.5	96.8
	BAC 39.7	37.3 ± 0.6	94.1	38.3 ± 0.6	96.5
Terra-Cortril	HYD 17.9	17.5 ± 0.2	97.9	17.1 ± 0.4	95.2
	OXY 53.6	54.2 ± 0.6	101.3	53.4 ± 0.4	99.7
Terra-Cortril ót-oftál	HYDA 59.2	61.5 ± 0.8	103.8	60.0 ± 0.4	101.3
	OXY 29.6	29.1 ± 0.5	98.2	30.7 ± 0.2	103.8
	PLX 80.4	80.8 ± 0.9	100.4	79.0 ± 0.9	98.2
Terramicina Tópica	OXY 60.0	58.2 ± 0.6	97.0	58.9 ± 0.5	98.1
	PLX 26.5	26.2 ± 0.2	98.9	25.8 ± 0.3	97.2

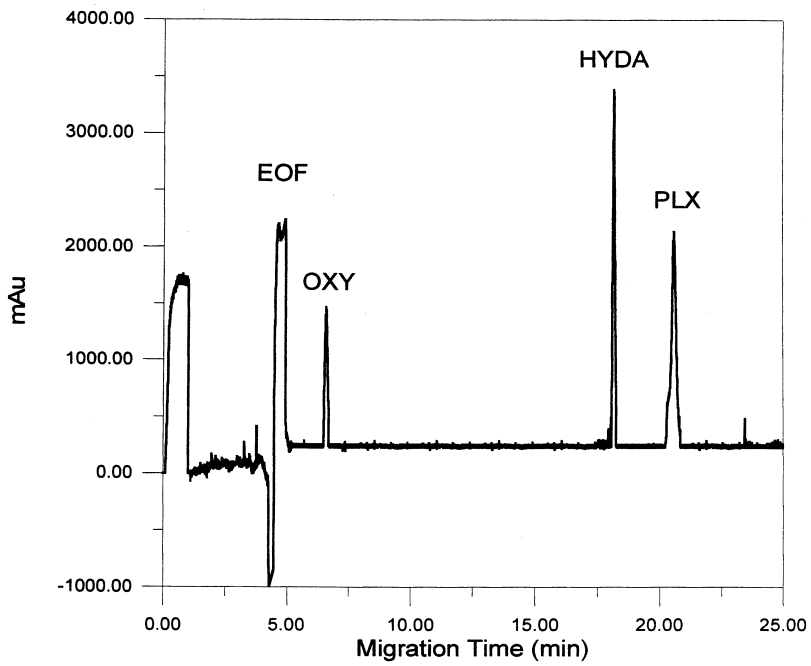

Figure 4. Electropherogram of Terra-Cortril ótico oftálmica obtained under the optimised conditions at 215 nm.

reproducibility results are comparable to those obtained with PLS-1 method, which comply with the requirements of drug quality control in terms of reproducibility and accuracy, and is also useful for routine analysis. In addition, it offers advantages such as simplicity of operation, flexibility, and low cost.

The new presented MEKC method to determine OXY, HYD, HYDA, PLX, and BAC was easy to apply to commercials, because there are no previous sample treatments, only a simple extraction of the commercials with methanol and a convenient dilution of the extract.

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