High-Performance Liquid Chromatographic Separation of Corticoid Alcohols and Their Derivatives: A Hydrolysis Study Including Application to Pharmaceuticals

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Abstract

Several reversed-phase high-performance liquid chromatographic methods for the baseline separation of some glucocorticoid alcohols and their derivatives (esters and acetals) have been developed. These methods involve fluorocortisone and fluorocortisone acetate, triamcinolone and triamcinolone acetonide, dexamethasone and dexamethasone phosphate, 21-hydroxydeflazacort and deflazacort, betamethasone and betamethasone valerate using deoxycorticosterone or methylprednisolone as an internal standard, a Hypersil C_{18} column, several mobile phases containing acetonitrile, and ultraviolet detection. These sensitive methods are used for a further hydrolysis study in an aqueous medium using standard solutions, which allowed the simultaneous monitoring of glucocorticoid derivatives and their hydrolysis products. In this study, the main variables that can affect the hydrolysis (pH and temperature) are also studied. Several applications to pharmaceuticals containing glucocorticoids are also reported.

Introduction

Glucocorticoids (naturally occurring or synthetic) are normally used to replace steroid hormones in patients with hormonal insufficiencies, as antiinflammatory drugs, and for diminishing the immunological response to a great variety of antigens, including transplanted organs. These drugs are normally synthesized either as glucocorticoid alcohols (GCAs) or derivatives of them (esters and acetals) (GCDs). The most common way to obtain a GCD is either by esterification with organic acids (e.g., valeric, acetic, or succinic) or inorganic acids (e.g., phosphoric) or by forming acetals from acetone (acetonides).

Several methods have been developed for the determination of a single GCA (1–4) and for GCDs in pharmaceutical preparations and biological fluids (5,6). However, little attention has been paid to the simultaneous determination of both GCDs and GCAs, even though it was required for different purposes such as pharmacokinetics studies in biological fluids and to investigate the stability of GCDs in vitro and in ampoules following long-term storage (7).

In this study, various high-performance liquid chromatographic (HPLC) methods are reported for the baseline separation of some GCDs and their GCAs as possible hydrolysis products (7). These separations were used for a further study on the stability of the GCD in an aqueous medium by controlling the most important variables of hydrolysis (time, pH, and temperature). This study was extented to several pharmaceutical preparations containing these compounds.

Experimental

Chemicals

Fluorocortisone (FL) (9α-fluoro-11β,17α,21-trihydroxypregn-4-ene-3,20-dione), fluorocortisone acetate (FLA), (fluorocortisone 21-acetate), triamcinolone (TRI) (9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione), triamcinolone acetonide (TRA) (9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17 acetal), dexamethasone (DX) (9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadien-3,20-dion), dexamethasone 21-phosphate (DXP), betamethasone (BM) (9α-fluor-11β,17α,21-trihydroxy-16β-methyl-1,4-pregnadien-3,20-dion), betamethasone 21-valerate (BMV), deoxycorticosterone (DOC) (4-pregnen-21-ol-3,20-dione), and methylprednisolone (MPL) (6α-methyl11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) were purchased from Sigma Chemical (St. Louis, MO). Deflazacort (DF) (11β,21-dihydroxy-2'-methyl-5'βH-pregna-1,4-diene[17,16-d]oxazole-3,20-dion 21-acetate) and its metabolite, 21-hydroxy-DF (DFM), were gifts from Marion Merrell Dow España (Madrid, Spain). Methanolic solutions of these compounds were prepared. All compounds were detected by HPLC with ultraviolet (UV) detection. Phosphoric acid of analysis grade was from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was from Promochem (Wesel, Germany). Water was purified with a Milli-Q system (Millipore, Molsheim,

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France). Millipore 0.45-µm nylon filters (Bedford, MA) were also used. All other chemicals were of analytical reagent grade.

Apparatus

The chromatographic system consisted of the following components, all from LDC Analytical (Riviera Beach, FL): a Constametric 4100 solvent delivery system, a spectromonitor 5000 photodiode-array detector covering the range of 190–360 nm and interfaced to a computer for data acquisition, and a recorder model CI 4100 data module. A Rheodyne 20-µL loop injector (Cotati, CA) and a Jones Chromatography block (heated series 7960) for controlling column temperature in the range of 30–60°C (Seagate Technology, Scotts Valley, CA) were also used. A Selecta (Barcelona, Spain) temperature-controlled water bath and a Digitronic Selecta temperature-controlled heater were used to control the hydrolysis temperature below and above 30°C ± 1, respectively. A reversed-phase Hypersil 5-ODS (250 × 4.6-mm i.d., 5 µm film thickness) column and a Spherisorb 5-ODS (10 × 4.6-mm i.d., 5 µm film thickness) guard column from Phenomenex (Torrance, CA) were also used.

Mobile phase

The mobile phase was prepared by mixing 1.2mM phosphoric acid (pH 3) with HPLC-grade acetonitrile in a required volume ratio by programming the pump. The acetonitrile and phosphoric acid solution was previously filtered under a vacuum through 0.45-µm nylon filters and degassed using helium sparge.

Chromatographic analysis

Once the column was conditioned with the mobile phase, chromatograms were obtained at a temperature of 25°C. Pure methanol containing a single corticosteroid or an appropriate mixture of them was injected (20 µL). The flow rate was 1.0 mL/min, and the UV–diode-array detection (UV–DAD) range used was 190–360 nm. Corticosteroids were identified by comparison between the UV spectra of the chromatographic peaks and UV spectra of previously registered standards. After identification, subsequent confirmation was performed by injection of each one. Analysis was carried out at 245 nm.

Hydrolysis of GCDs and HPLC analysis

Hydrolysis of GCDs was carried out in the temperature range of 20–50°C. Solutions containing GCDs (10 µg/mL) and the internal standard (IS) (5 µg/mL) were prepared in various aqueous media: phosphoric acid (pH 3), acetate buffer (pH 4.7), phosphate buffer (pH 7.2), and sodium hydroxide (pH 9.3 and 12). Samples were analyzed in the range of 1 min–3 h. For shorter reaction times, hydrolysis was stopped by adding concentrated phosphoric acid; for longer reaction times, the mobile phase containing phosphoric acid (pH 3) was used. GCD hydrolysis and GCA formation were assessed by plotting the peak area ratio (PAR) of the GCD or GCA to the IS (DOC or MPL) versus time.

Table I. Retention Factors for GCDs and GCAs Obtained with Acetonitrile–Phosphoric Acid Solution (pH 3) Mobile Phases (% v/v) and MPL and DOC ISs

Compounds	H_3PO_4 solution (pH 3)	Acetonitrile	Retention factor (k)
FL			2.52
FLA	55	45	6.37
MPL			3.04
DFM			4.83
DF	60	40	7.60
MPL			3.35
BM			2.30
BMV	45	55	7.95
DOC			5.01
TRI			2.32
TRA	68	32	15.05
MPL			9.62
DXP	Gradient: 10, 10, and 40% AcN in		3.42
DX	0, 12, and 20 min		14.85
MPL			14.23

Sample preparation

Pharmaceutical samples such as injectables, tablets, creams, ointments, and coliriums containing GCDs were employed and processed by triplicate. Injectables (Trigon Depot) containing 40 mg/mL TRA were diluted adequately with 20mM phosphate buffer (pH 7.2) to obtain two separate 5-µg/mL solutions. Coliriums (Colircusi DX) containing 1 mg/mL of DXP were adequately diluted with 20 mM phosphate (pH 7.2) to obtain two separate 10 µg/mL solutions. Tablets (Dezacor, 30 mg) containing DF were adequately pulverized. The fine powder was weighed in the amount of 0.37 g and adequately diluted with phosphoric acid solution (pH 3.0) or phosphate buffer (pH 7.2) to obtain two separate 8-µg/mL solutions. Creams (0.6 g Celestoderm V) containing 0.5 mg/g BMV or ointments (0.3 g Fludronef) containing 1 mg/g FLA were treated with 3 mL of 20mM acetate buffer (pH 5.5) and adequately diluted to obtain separate 8-µg/mL solutions. The above samples contained 5 µg/mL of an IS (see Table I).

Results and Discussion

Separation of GCDs in the presence of GCAs

It is known from the literature that some GCDs are vulnerable to chemical and enzymatic hydrolysis, giving GCA compounds (7–9). For this reason and in order to undertake a further hydrolysis study of GCDs in an aqueous medium, several simultaneous baseline separations involving GCD and GCA compounds using an IS (MPL or DOC) have been carried out. On the basis of other chromatographic studies previously developed (10–11) involving similar corticoids and several mobile phases, acetonitrile was selected as an organic modifier and optimized at a column temperature of 25°C. Table I shows a

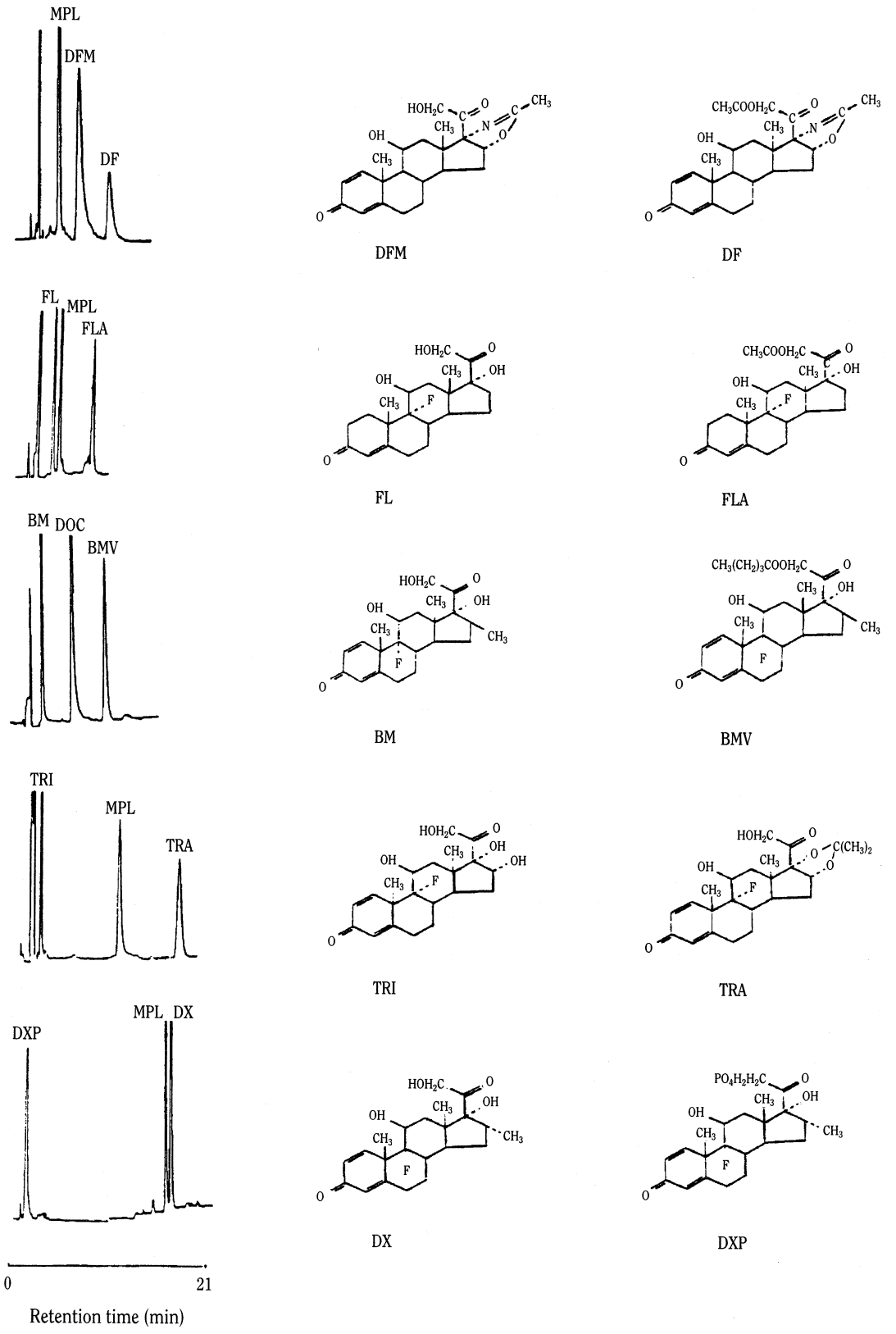

Figure 1. Chemical structures and HPLC separations involving GCD and GDA compounds. Mobile phases are listed in Table I.

summary of the mobile phases used and the values of the retention factors (k) obtained. The chemical structures and the chromatograms obtained for these separations are shown in Figure 1. These separations have the advantage of monitoring both GCD and GCA compounds. The broadening of DF and DFM peaks was probably due to interactions between the column (silanol groups) and nitrogen-containing compounds.

Calibration graphs

Standards containing mixtures of GCDs, GCAs, and the IS (Table I) were prepared at five different concentrations in the range of 2–10.0 µg/mL using 5.0 µg/mL IS (MPL or DOC). These solutions were analyzed with the mobile phases summarized in Table I using a flow rate of 1.0 mL/min, a Hypersil column, and UV–DAD at 245 nm. Plotting each corticosteroid peak area to the IS ratio (PAR) versus the concentration (x), the calibration equations were obtained (PAR = $A + Bx$ [µg/mL]). In Table II, the parameters A (intercept), B (slope), and r (regression coefficient) are shown. In all cases, the intercepts were not significantly different from zero.

Precision and detection limits

The precision was examined by analyzing 10 different samples of corticosteroids containing 6 µg/mL each by means of the calibration graphs. The relative standard deviation (RSD) for the corticoids under study is shown in Table II. The detection limits obtained for a signal-to-noise ratio of 3 are also in Table II.

Preliminary hydrolysis test: GCA identification

To obtain preliminary information about the hydrolysis of GCDs, some experiments were performed under various pH conditions (pH 3–12). In such conditions, standards were maintained for 24 h at a reaction temperature of 25°C. For product identification, retention times and UV absorption spectra obtained from chromatographic peaks on Figure 1 were then compared with those obtained after reaction. In this way, all the proposed GCAs were identified. Exception was made with regard to TRA because two compounds were rendered: TRI and an unknown compound with a UV spectrum similar to those of other glucocorticoids (OGC) with a higher retention time than TRI (more hydrophobic). This behavior suggests a double reaction mechanism involving a dihydroxy- (TRI) and a hydroxy- compound (OGC) (Figure 1). For quantitative assessment of the hydrolysis products, samples were analyzed using the separations previously obtained (Table I and Figure 1) and calibration graphs.

The experimental data obtained from this study are summarized in Table III. As can be observed, the hydrolysis did not occur at pH 3 but was favored in the alkaline medium, except DXP, which was hydrolyzed at pH 4.7. From these experiments, it could be concluded that the hydrolysis might be stopped either with mobile phases containing phosphoric acid (pH 3) or by adding this acid to the reaction mixture (see *Hydrolysis of GCDs and HPLC analysis* section).

Table II. Linear Regression Equations* of GCD and GCA

Compound	A	B	Correlation coefficient	Detection limit (ng)	RSD (%)
FL	0.004	0.278	0.998	0.04	1.3
FLA	0.030	0.222	0.998	0.05	2.8
DFM	0.021	0.136	0.999	0.08	3.0
DF	0.033	0.133	0.998	0.08	2.7
BM	0.032	0.101	0.999	0.08	3.4
BMV	–0.002	0.080	0.997	0.06	1.5
TRI	–0.012	0.132	0.999	0.08	1.6
TRA	0.002	0.077	0.999	0.13	0.8
DXP	–0.027	0.107	0.990	0.09	2.8
DX	–0.066	0.150	0.999	0.06	1.5

* Peak area ratio = $A + Bx$, where x is the concentration (µg/mL) of GCD or GCA.

Table III. Hydrolysis Percentage over a Test for GCD at Different pH Values*

GCD	GCA	pH values				
		3.0	4.7	7.2	9.3	12.0
TRA	TRI + OGC	0.0	0.0	0.0	0.0	33
FLA	FL	0.0	1.3	58	65	100
DXP	DX	0.0	7.9	0.0	0.0	0.0
BMV	BM	0.0	0.8	1.0	13	100
DF	DFM	0.0	0.4	1.7	7.0	100

* 25°C and 24-h reaction time.

Table IV. Hydrolysis Percentage and Recoveries for Pharmaceuticals Containing GCD under Different Physiological Conditions of Temperature (37°C) and pH*

Pharmaceuticals	GCD recovery (%)	pH	Hydrolysis (%)
Injectables	TRA (100)	7.2	0.0
Tablets	DF (99 ± 2.1)	3.1	0.0
		7.2	1.5
Creams	BMV (98 ± 2.6)	7.2	0.0
Ointments	FLA (53 ± 3.4)	7.2	51
Coliriums	DXP (100)	7.2	0.0

*See also Table III.

Table V. Hydrolysis Percentage versus Time Obtained for Samples Containing Fluocinolone Acetonide at 37°C and pH 7.2

Time (h)	Hydrolysis (%)
0	0
12	5
24	10
36	35
48	50

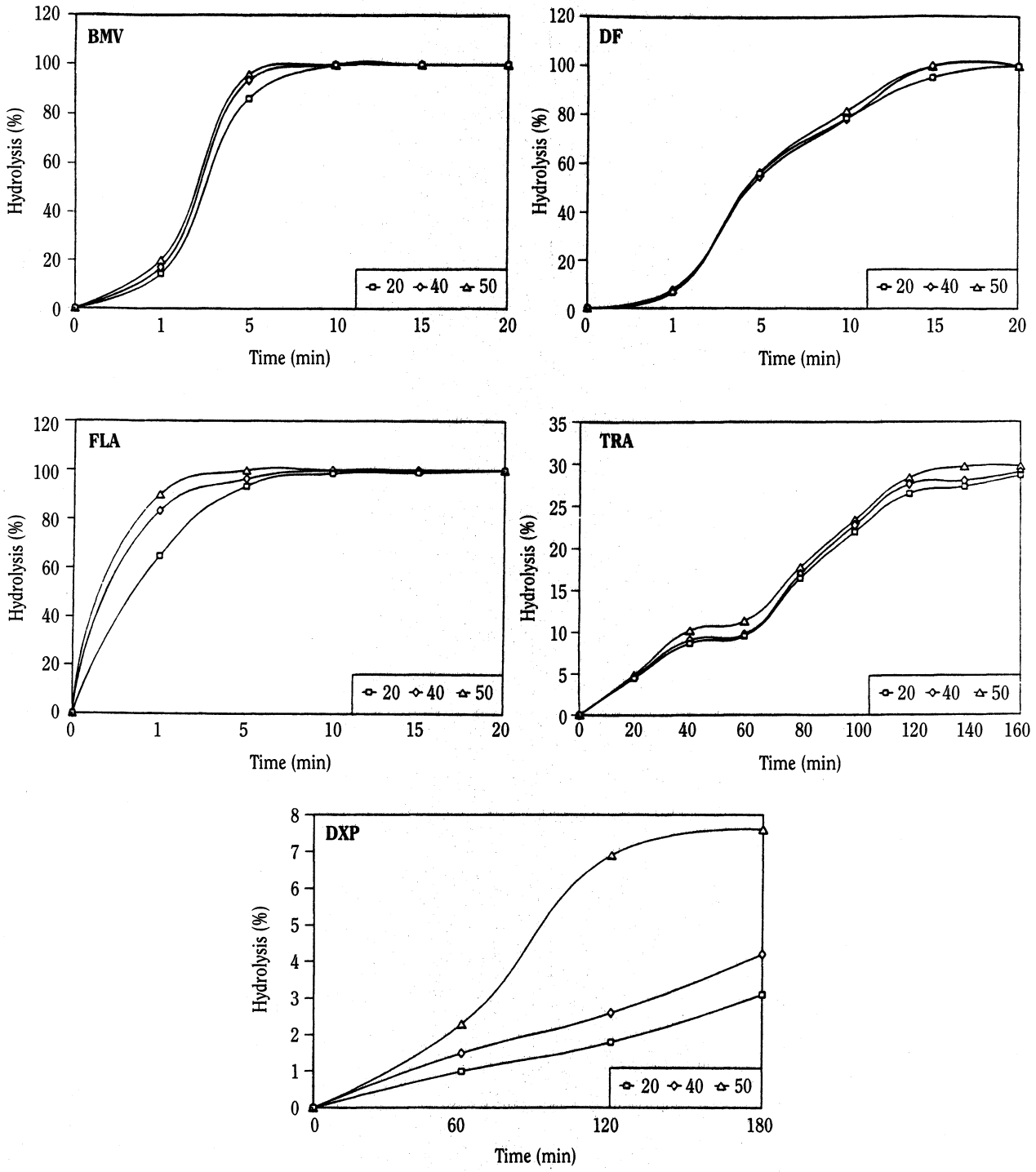

Figure 2. Effect of temperature on the hydrolysis of GCDs: BMV, DF, FLA, and TRA at pH 12 and DXP at pH 4.7. Temperatures shown: 20, 40, and 50° C.

Influence of hydrolysis variables

Based on the above test, the hydrolysis of standard solutions containing GCDs was studied considering the main variables that can affect these reactions (time, pH, and temperature). These effects were only studied when the hydrolysis was positive (Table III). Peak area ratios of the consumed GCD or the produced GCA to the IS (DOC or MPL) were obtained under different hydrolysis conditions. After this, the hydrolysis percentage (% H) was obtained from calibration graphs.

The effect of temperature on GCD hydrolysis was studied in the range of 20–50°C at pH 12 for all GCDs except DXP and at pH 4.7 for DXP only. As can be observed in Figure 2, all GCD

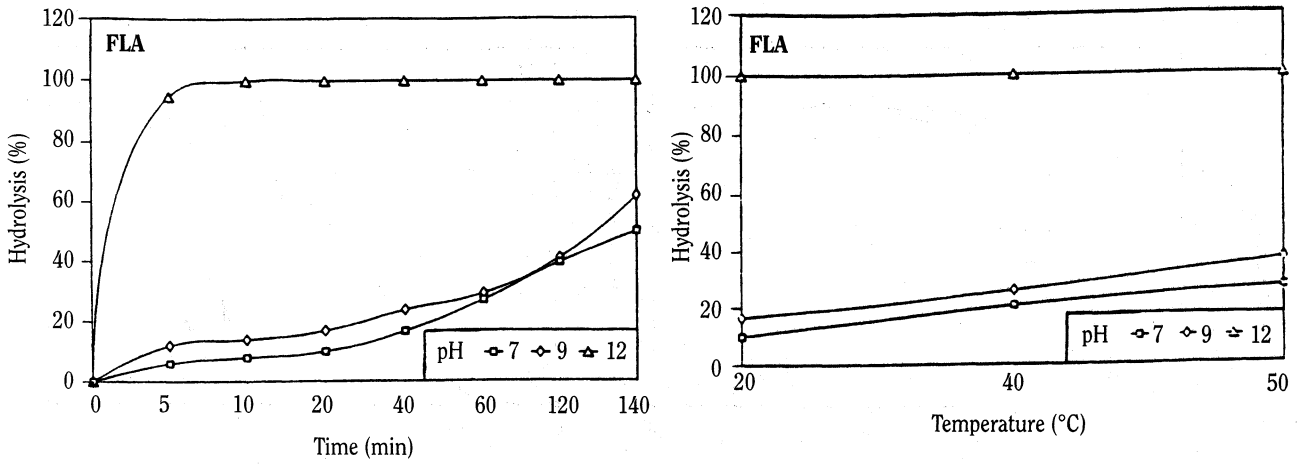

Figure 3. Effect of pH on FLA hydrolysis. (A) Hydrolysis versus reaction time (40°C) at different pH values. (B) Hydrolysis versus temperature at different pH values (reaction time, 20 min).

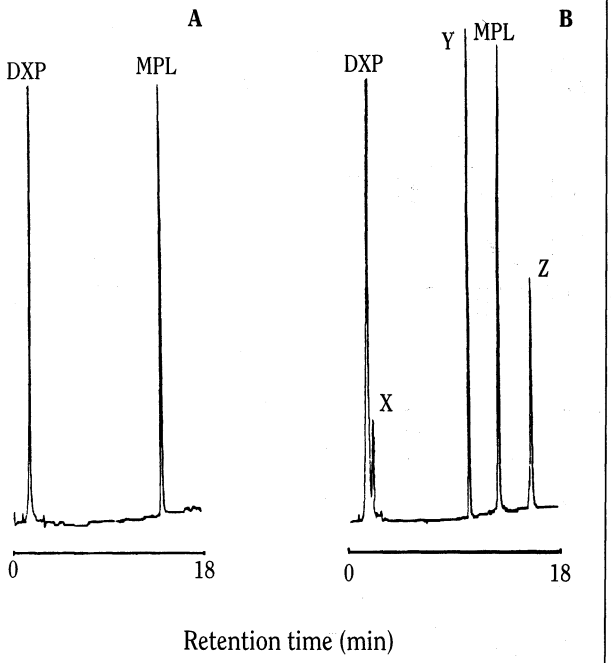

Figure 4. Chromatograms obtained from two separate colirium samples containing DXP: (A) fresh and (B) prepared approximately 2 years ago. Mobile phases are listed in Table I.

compounds were affected by temperature. Hydrolysis increased when the temperature was increased. At 50°C, quantitative results were achieved for BMV (5 min), FLA (5 min), and DF (15 min). However, it was incomplete for TRA and DXP (to obtain 30% hydrolysis for TRA and 7% for DXP, 160 min was required).

The effect of pH was also studied (25°C) in the range of 3.0–12. Hydrolysis was observed in the pH range of 4.7–12 for FLA, BMV, and DF. However, TRA and DXP only afforded hydrolysis at pH 12 and 4.7 (Table III). Figure 3 shows the FLA behavior versus hydrolysis as an illustrative example. As can be observed (Figure 3A), hydrolysis (40°C) increased with pH (e.g., at 20 min, quantitative hydrolysis was obtained at pH 12, but not at a lower pH). Figure 3B shows the effect of temperature at a fixed time (20 min). At any pH value, hydrolysis increased as temperature increased. These results are in agreement with those obtained for similar compounds (7).

Stability studies of GCDs in pharmaceuticals

Based on the above separations and hydrolysis tests, in vitro stability studies on different pharmaceutical preparations (Table IV) containing GCD compounds were carried out. From these data, physiological conditions (37°C) were selected. Hydrolysis reactions were assessed (maximum 48 h) from chromatographic data obtained by comparing samples under hydrolysis and nonhydrolysis conditions. In this way, the presence of interferences was tested. Table IV also summarizes the recoveries found (under sample preparation conditions) and the hydrolysis percentage obtained from pharmaceuticals containing GCDs. Table V shows the hydrolysis percentage versus time for FLA at pH 7.2. As can be observed, hydrolysis increased as time increased.

Figure 4 shows the chromatograms obtained from two samples containing DXP (colirium) previously stored at room temperature. One sample was used approximately 2 years ago, and the other was a fresh colirium. With regard to the old preparation, compounds Y (maximum wavelength, 200 and 251 nm) and Z (maximum wavelength, 256 nm) (Figure 4B) were preservative compounds containing samples that were not present in the new one (Figure 4A). Moreover, compound X presented the characteristic absorption spectrum of corticosteroids (maximum wavelength, 245 nm). This compound did not correspond to DX (see Figure 1). As expected, DXP hydrolysis did not take place at pH 7.2. However, a more hydrophilic compound than DX was obtained (Figure 4B). This unknown compound may have been due to an impurity derived from the DXP synthesis or derived from an unknown degradation process.

Samples containing DF (tablets) were also investigated. As expected, chemical hydrolysis in the stomach or duodenum for tablets practically did not take place. However, DF is converted

to DFM by blood seric sterases (11). Other investigated samples (Table IV) gave no positive results.

Conclusion

Different efficient chromatographic methods for pairs of corticoids have been developed that allow the simultaneous monitoring of GCDs and their hydrolysis products in a sensitive and selective way. These methods hold special interest in their application to the analysis of different pharmaceutical preparations with different purposes, such as long- and short-term stability and quality control.

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