Journal of Chromatography, 78 (1973) 165–171

CHROM. 6479

SEPARATION AND DETERMINATION OF THE SYNTHETIC EPIMERIC CORTICOSTEROIDS DEXAMETHASONE AND BETAMETHASONE IN MIXTURES BY THIN-LAYER CHROMATOGRAPHY ON INSOLUBLE POLYVINYLPYRROLIDONE

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SUMMARY

A thin-layer chromatographic method suitable for the determination of the two epimeric corticosteroids dexamethasone and betamethasone in mixtures is described. The procedure is based on the use of insoluble polyvinylpyrrolidone layers and anhydrous acid solvents. By means of the isonicotinic acid hydrazide fluorescence reagent, quantitation is possible by direct fluorimetry using multiple standard spots as reference points. By using this technique, up to 5 % of each epimer in the presence of the other can be accurately determined. Paramethasone and other hydroxycorticosteroids are also well separated under these conditions.

INTRODUCTION

In recent years, thin-layer chromatographic (TLC) methods have been extensively used to identify, to separate and even to determine synthetic corticosteroids that are useful in topical and systemic anti-inflammatory therapy. Excellent TLC procedures applicable to the analysis of different drugs have been developed, mainly those that differ from each other by the presence or absence of polar groups, but certain problems remain unsolved although they are of no less importance from the pharmaceutical standpoint.

One of the most outstanding problems is that of the characterization of slightly polar and non-polar functions in the same basic structure (*e.g.*, double bonds, fluorine atoms, methyl and methylene groups) and especially that of certain spatial structures, such as α-methyl and β-methyl. Difficulties arising in these instances have frequently been discussed in the pharmaceutical and biochemical literature[1–12], as they concern important groups, in particular in 11-hydroxy-corticosteroid derivatives.

The separation and determination of two important isomers (epimers), namely dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione) and betamethasone (9α-fluoro-16β-methyl-11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione) have proved to be especially difficult. Also, although less important, the separation and determination of parametasone (6α-fluoro-16α-methyl-11β,17α,21-

trihydroxy-pregna-1,4-diene-3,20-dione) in the presence of the above two compounds are in some instances a significant problem.

The chromatographic differentiation of these isomers in their mixtures is difficult when the substrates and solvents that are usually recommended for TLC procedures are used[6–8,10,13] except, perhaps, when more complex techniques are used[7,14], in which event both of the advantages of TLC — speed and simplicity — are unfortunately lost to a great extent. Recently, the U.S. National Formulary XIII has included in its monographs a TLC procedure for identifying dexamethasone and betamethasone[15], which, however, must be considered to be unsuitable for the separation and determination of these corticosteroids when they are present in a mixture.

In a previous paper[16], we reported a TLC method suitable for the separation and identification of hydrocortisone (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione), prednisolone (11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione) and their 9α-fluorinated homologues. The procedure was based on the use of insoluble polyvinylpyrrolidone layers and aqueous solvents as developing fluids. In the present paper, the results obtained on the same substrate when using anhydrous solvent systems by means of a more simple technique are reported. By this method, the epimeric corticosteroids dexamethasone and betamethasone can be identified and determined in their mixtures and the separation of paramethasone from these two isomers can also be achieved.

MATERIALS AND METHODS

Commercially available Polyclar AT (General Aniline and Film Corporation, New York, U.S.A.), a type of internally crosslinked polyvinylpyrrolidone, which is insoluble in water, organic solvents, acids and alkalis, was used. The commercial product was screened through a set of A.S.T.M. sieves in a vibrating apparatus (Endicott type), and the fraction of particles between 37 and 53 μm was selected and used without any further treatment.

Of the selected powder, 6 g were dispersed in 37.5 ml of anhydrous isopropanol and stirred by hand for about 1 min. The resulting suspension was distributed on five 20 × 20 cm glass plates so that a layer thickness, when wet, of 250 μm was obtained by means of the Stratomat Chemetron automatic apparatus. The plates were air-dried overnight and used directly for chromatography. The adherence and stability of the layers were satisfactory.

Qualitative tests

Solutions (0.01 and 0.1 % w/v) of the pure corticosteroids in a mixture of equal volumes of methanol and chloroform were used for spotting with the aid of a Link micropipette. Amounts of 1–20 μg were generally used.

After the laborious testing of several solvent systems, a 94:6 (v/v) mixture of dichloromethane and acetic acid was selected as the most suitable developing fluid. The development was carried out in 21 × 21 × 9 cm Desaga chambers at 20 ± 1° in the usual way. The chamber was equilibrated with the solvent for not less than 12 h by lining the side-walls with filter-paper soaked with solvent. The total development time was about 45 min for a length of run of 15 cm. The chromatograms were air-dried at room temperature.

A 100-ml volume of a 0.4 % (w/v) fresh solution of isonicotinic acid hydrazide (INH) in anhydrous methanol was prepared and 0.5 ml of concentrated HCl was added[17]. This mixture was used as a spray-reagent for the detection of the spots. After spraying, the plates were maintained at room temperature for 24 h, after which yellow spots with bright yellow fluorescence under 367-nm UV irradiation appeared (Fig. 1). The sensitivity was excellent, even for amounts that were ten times less than the minimum mentioned above.

Alternatively, the Tetrazolium Blue reagent, prepared as described earlier[16] was used for qualitative purposes (Fig. 2).

The mobility of the solutes was expressed as R_F values. The standard deviation of the mean R_F values was calculated statistically by means of a Programma 101 (Olivetti) computer.

Determinations

The spots were quantified by means of a Vitatron TLD 100 Hg flying-spot densitometer, with a mercury lamp as a light source, using as the reference point the integration numbers obtained in a Vitatron UR 402 recorder. The instrument settings were as follows: level, F; zero (c), 7; damping, 3; diaphragm, 2.5 × 0.25; mode, linear II positive; primary filter, 367 nm; secondary filter, 567 nm; amplitude,

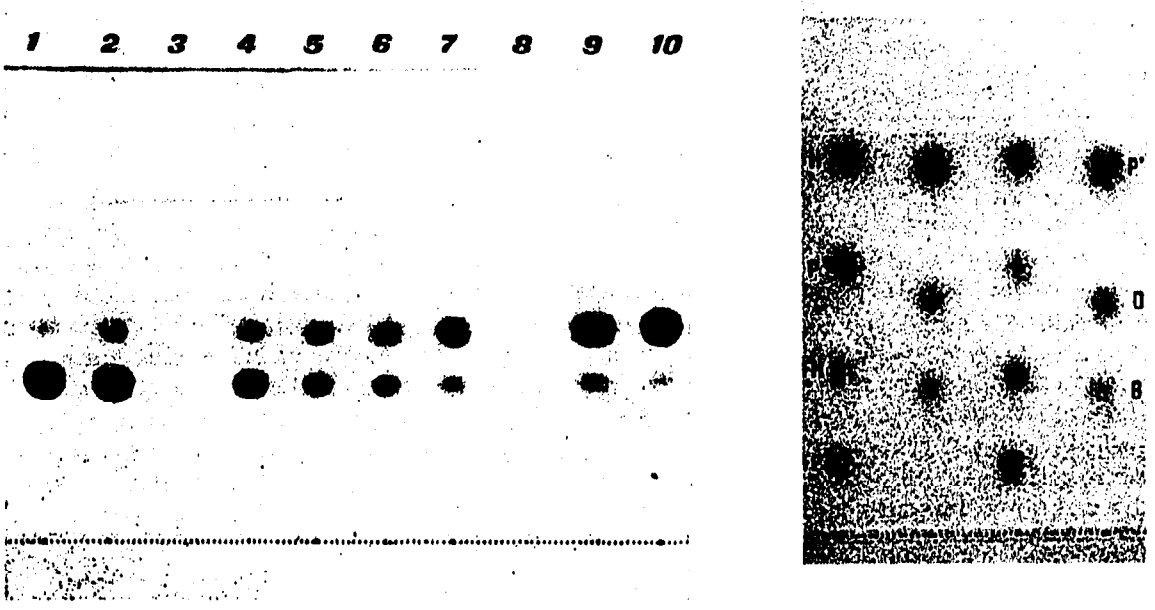

Fig. 1. Experimental chromatograms of standard mixtures of dexamethasone and betamethasone on insoluble polyvinylpyrrolidone plates showing the procedure recommended for quantitative purposes. Whereas lanes 3 and 8 are reserved for the unknown mixture, the standard solutions (5 μl) are spotted as given in Table I. Length of run, 15 cm. Detection reagent, isonicotinic acid hydrazide (INH)[17]. Photograph obtained under UV irradiation at 367 nm.

Fig. 2. Chromatogram of two mixtures of different 11-hydroxycorticosteroids on polyvinylpyrrolidone plates. Length of run, 15 cm. Detection reagent, Tetrazolium Blue[16]. H = hydrocortisone; P = prednisolone; FH = fluorohydrocortisone; FP = fluoroprednisolone; P' = paramethasone; D = dexamethasone; B = betamethasone.

TABLE I

STANDARD SOLUTIONS (MIXTURES OF FIXED, VARIABLE AMOUNTS OF DEXAMETHASONE AND BETAMETHASONE) USED FOR QUANTITATION

A 5-μl volume of each solution was spotted on to the corresponding lane on a polyvinylpyrrolidone plate using a 10-lane template (see Fig. 1). Lanes 3 and 8 were reserved for the unknown, prepared by dissolving 4 mg of the sample steroid or the weighed residue of the extract into 1 ml of methanol–chloroform (1 : 1). A 5-μl volume of the resulting solution was spotted, corresponding theoretically to 20 μg of total steroids. The plate was developed and detected by spraying with INH fluorescence reagent. After quantitation in the Vitatron apparatus, the integration numbers obtained for the unknown were interpolated on the corresponding calibration curve, between the two adjacent standard points.

Standard solution spot number	*Initial concentrated solutions in methanol–chloroform (1 : 1)*				*Methanol–chloroform solvent (ml)*	*Amount of steroid in spots (μg)*	
	Betamethasone 1% (ml)	*Dexamethasone 0.1% (ml)*	*Betamethasone 0.1% (ml)*	*Dexamethasone 1% (ml)*		*Betamethasone*	*Dexamethasone*
1	4	2	—	—	4	20	1
2	3	4	—	—	3	15	2
3[a]	—	—	—	—	—	—	—
4	2	6	—	—	2	10	3
5	1	8	—	—	1	5	4
6	—	—	8	1	1	4	5
7	—	—	6	2	2	3	10
8[a]	—	—	—	—	—	—	—
9	—	—	4	3	3	2	15
10	—	—	2	4	4	1	20

[a] No standard solutions. In lanes 3 and 8 the unknown mixture is spotted.

10 (5 mm); paper velocity, 5 (2 cm/min); integrator, 7 (60 counts/min/cm); span, 6.7; and scanning speed, 2 (1 cm/min).

As the plate-to-plate variations observed between standard integration numbers were relatively large, the following technique was established for the quantitation of the spots. A series of standard solutions containing increasing or decreasing amounts of the steroids to be determined (dexamethasone and betamethasone) was prepared and each mixture labelled as indicated in Table I. By means of a ten-point template, 5 μl of these solutions were spotted on a polyvinylpyrrolidone plate with a Chemetron microsyringe in the following manner: two lanes, namely 3 and 8, were reserved for the unknown mixture, and the remaining eight lanes (1, 2, 4–7, 9 and 10) were used for the standards, ranging from 1 to 20 μg of each steroid. The plate was then developed and the spots were detected by spraying with INH reagent. Between 24 and 96 h after spraying, the layers were scanned and the integration numbers for each spot recorded.

As the integration numbers can be considered to be linearly related to the amounts chromatographed only within a relatively small range of concentrations, more than one calibration curve is necessary for each steroid when the concentration range used is too large, so that the determination of a substance of unknown concentration in a particular spot could be readily achieved simply by interpolation. By using this technique, the determination of the components in an unknown mixture

can be achieved by using only one chromatogram, as indicated in the section RESULTS. This procedure is suitable for mixtures of dexamethasone and betamethasone. Although this is the only mixture that we tested, nevertheless, as these two corticosteroids have the most similar R_F values, it is probable that the determination of paramethasone in the presence of these two isomers could also be readily achieved.

RESULTS

The spots were circular, without any tailing, as can be seen in Figs. 1 and 2. The R_F values obtained for the three corticosteroids qualitatively tested (mean of 20 chromatograms on different plates) as well as the corresponding R_M values, are given in Table II. It can be seen from the small standard deviation that the reproducibility under the conditions used was satisfactory.

TABLE II

MEAN R_F VALUES AND OTHER CHROMATOGRAPHIC PARAMETERS FOR BETAMETHASONE, DEXAMETHASONE AND PARAMETHASONE ON POLYVINYLPYRROLIDONE PLATES

Results obtained on polyvinylpyrrolidone plates using dichloromethane–acetic acid (94:6) as eluent for a length of run of 15 cm (development about 45 min). Temperature, 20 ± 1°.

Steroid	$R_F \times 100$	*S.D.*	R_M	ΔR_M	*Functional group*
Betamethasone	30.5	±1.05	+0.357		
				+0.259	16β-CH_3 → 16α-CH_3
Dexamethasone	44.4	±1.09	+0.098		
				+0.458	9α-F → 6α-F
Paramethasone	69.4	±2.32	−0.360		

The ΔR_M value (16β-CH3 → 16α-CH3) is sufficiently large to separate adequately dexamethasone and betamethasone in an unknown mixture. The ΔR_M value (9α-F → 6α-F), being larger than the former, permits the excellent separation of paramethasone from the other two compounds. In general, the reported results not only permit complete separation but also enable these three closely related steroids to be easily identified in any mixture of them merely by comparing their relative migration characteristics.

As mentioned, the determinations were carried out only on dexamethasone–betamethasone mixtures. The operating method described above is summarized in Table I and Fig. 1.

For the quantitative analysis of any unknown mixture it is recommended to place 5 μl of the solution to be analyzed (a 0.4 % solution of the labelled steroid or of the weighed extracted product or residue) on lanes 3 and 8. After development and detection, the chromatogram is quantified and the integration numbers obtained for the sample, containing 20 μg (theoretical) of total steroids, are compared with the calibration curve.

By means of this procedure, up to 5 % of each corticosteroid in the presence of the other can be accurately determined. Statistical analysis of the results has shown good to excellent correlation coefficients for the tested mixtures. A typical photodensitogram corresponding to the calibration curves drawn in Fig. 3 is shown in Fig. 4.

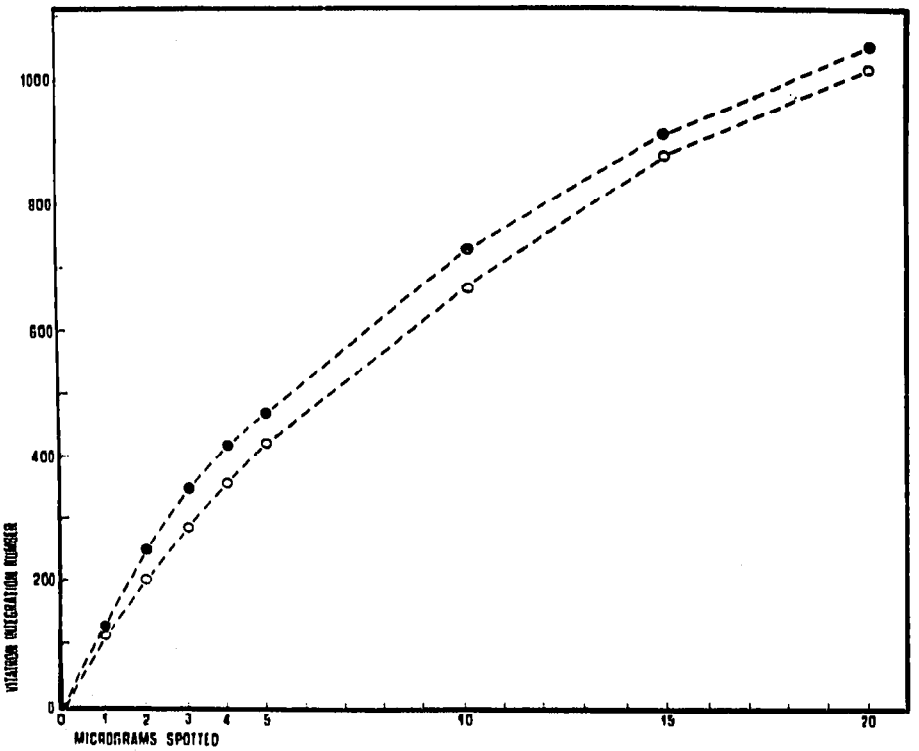

Fig. 3. Calibration curves relating Vitatron integration numbers and amounts of corticosteroids in the standard spots, drawn according to the data shown in Fig. 4. ●—●, dexamethasone; ○—○, betamethasone.

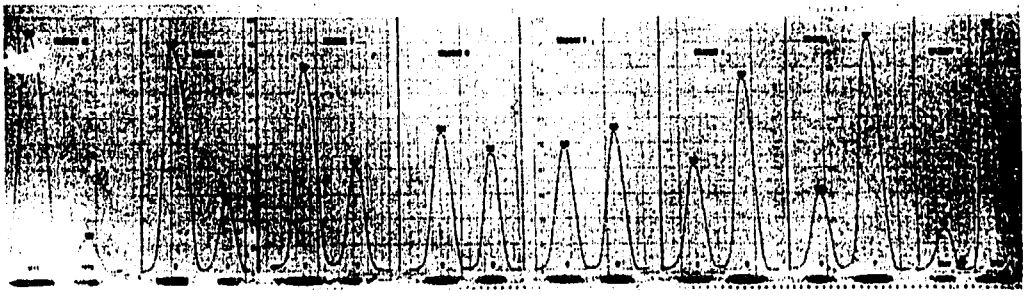

Fig. 4. Photodensitogram obtained in the Vitatron apparatus for the standard mixtures of dexamethasone and betamethasone shown in Fig. 1 (see also Table I). Integration numbers are indicated above the curves.

DISCUSSION

Attempts to separate dexamethasone and betamethasone from their mixtures by means of the conventional TLC methods have been unsuccessful apart from one outstanding exception.

Although markedly different in most features, *e.g.*, their relatively hydrophobic character, polyamide substrates are, as polyvinylpyrrolidone substrates, strong proton-acceptor adsorbents[18, 19]. Success in finding anhydrous solvents suitable

for achieving good separations for the above and other closely related steroids on polyvinylpyrrolidone plates led us to retest some polyamide layers that were unsuitable when aqueous solvents were used[20]. It was found that by using Polyamide F-254 (Merck) plates and proton-donor solvents, such as dichloromethane or chloroform (alone or with very small proportions of low-molecular-weight alcohols), satisfactory separation of dexamethasone and betamethasone was obtained, as-shown by R_F values of 0.38 for dexamethasone and 0.53 for betamethasone (in dichloromethane).

However, none of these chromatograms could be adequately quantified owing to difficulties in the detection of the spots. The direct evaluation of the chromatograms by quenching of the UV fluorescence at 254 nm also failed owing to the lack of sensitivity, even when relatively large amounts of each steroid are present. Further work in order to quantify the spots obtained on polyamide plates is in progress.

From the results obtained so far, we have concluded that insoluble polyvinylpyrrolidone has proved to be the most suitable chromatographic substrate for the separation, identification and determination of these and other 11-hydroxy-corticosteroids (21-alcohols and 21-acetates) in their mixtures. Fig. 2 shows a chromatogram in which the outstanding features of the substrate in separating some closely related 11-hydroxy-corticosteroids in mixtures can be appreciated.

ACKNOWLEDGEMENTS

The expenses of this work were defrayed by a grant from the University Investigation Fund (F.I.U.) from the Ministry of Education and Science.

We thank Mr. FREDY RUIZ for valuable technical collaboration during the development of this work.

We thank Schering Corp. (Bloomfield, U.S.A.), Ely Lilly and Co. (Indianapolis, U.S.A.), Merck AG (Darmstadt, G.F.R.) and Upjohn Co. (Kalamazoo, U.S.A.) for supplying the samples of pure corticosteroids.

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