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Quantitative TLC Determination of Epimeric Ratios of 16-Methyl 17-Ketone Oxidation Products of Dexamethasone and Related Drugs

E. C. JUENGE ˣ and P. E. FLINN

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Abstract □ A TLC system sensitive enough to detect, identify, and allow quantitation of the 16-methyl α- and β-epimers of 9-fluoro-11β-hydroxy-16-methylandrosta-1,4-diene-3,17-dione is described. These epimeric 17-ketones may be present as impurities in dexamethasone, betamethasone, or related drugs. A spectrodensitometer with a TLC scanning attachment was used, and results from densitometry compared favorably with quantitation by high-performance liquid chromatography, as was described recently. TLC was convenient in the rapid examination of drug samples for the detection and identification of epimeric 17-ketones and for the determination of the α to β ratio of such epimers. Various applied photographic techniques for documenting TLC data are described.

Keyphrases □ TLC—detection and identification of 16α- and 16β-methyl epimers of 9-fluoro-11β-hydroxy-16-methylandrosta-1,4-diene-3,17-dione, impurities in dexamethasone, betamethasone, and related drugs □ Dexamethasone impurities—16α- and 16β-methyl 17-ketone oxidation products, identification and quantitative detection by TLC □ Epimers—16α- and 16β-methyl 17-ketone oxidation products, possible impurities in dexamethasone, betamethasone, and related drugs, TLC detection and identification of α- and β-epimers

The TLC or partition chromatography of C_{18} steroid estrogens (1–3), C_{19} androstanes (4–6) such as androgenic hormones, C_{21} pregnanes (7–10) including cortisone, and the related oxygenated derivatives of these families is documented. The C_{22} steroids such as dexamethasone and betamethasone and their C_{20} oxidation products have not received such detailed study.

Interest has developed in qualitative and quantitative studies of these epimeric C_{20} oxidation products by high-performance liquid chromatography (HPLC) (11) and TLC (12) because of their presence as impurities in dexamethasone (11–13) and dexamethasone sodium phosphate (I) drug samples. Inherently, the C_{22} steroids and their 17-oxo derivatives possess a structural feature not exhibited by the C_{19} and C_{21} steroids and their oxygenated derivatives, namely, an optical center resulting from the additional methyl group at the C-16 position. These epimeric optical isomers were subjected to HPLC and TLC in this study.

BACKGROUND

The 17-oxo derivative, *i.e.*, 9-fluoro-11β-hydroxy-16-methylandrosta-1,4-diene-3,17-dione (epimers IIα and IIβ), recently was reported to be present (12) at ~2% as an impurity in drug products from five of six dexamethasone manufacturers for the Canadian market. In addition, a massive contamination (50%) by the α- and β-epimers of this ketone (IIα and IIβ) was reported (11) in a commercial sample of dexamethasone sodium phosphate (I) solution for injection. In that study (11), it was found by HPLC that this 17-keto oxidation product was actually an epimeric mixture of the 16α- and 16β-methyl compounds and that the α to β ratio could be determined approximately. Identification of the 16-methyl 17-ketone epimers (IIα and IIβ) by TLC systems similar in nature to the reversed-phase HPLC system was attempted, but the TLC systems failed to separate the α- and β-epimers; dexamethasone acetate, a related compound, consistently exhibited the same R_f value as the epimers (11).

Another report (12) indicated that separation between the 16α- and 16β-methyl epimers (IIα and IIβ) can be effected by TLC (R_f 0.60 and 0.59, respectively). The epimeric corticosteroids, dexamethasone and betamethasone, which differ in configuration at the C-16 optical center, were separated by paper chromatographic liquid partition (14). General techniques for photographic documentation of data collected on TLC plates were reported (15).

A method to determine the α to β epimeric ratio of the impurity is important because the components of the impurity are diastereoisomeric with different physiological properties and, consequently, different effects on the patient. An overview of possible physiological effects of such contamination, including references, was given previously (11).

The aim of the present work was to develop a TLC technique that allows identification of epimeric 16-methyl 17-ketones (IIα and IIβ) in samples of drugs related to dexamethasone. Furthermore, the method must determine the α to β ratio of the impurity. In the first part of the

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study, the TLC method was evaluated as a qualitative tool for detection of the epimeric 16-methyl 17-ketones in drug samples submitted to this laboratory for quality assurance studies; such a method must be convenient and flexible, including reliable procedures for documentation of results. In the second part, the suitability of the TLC method was investigated for quantitative measurement of the epimeric ketones.

EXPERIMENTAL

Instruments—A spectrodensitometer[1] with a TLC scanning attachment and basic computing readout system, a UV–near-IR spectrophotometer[2], and a grating IR spectrophotometer[3] were used. Melting points were taken with a microscopic[4] melting-point stage[5]. An analytical balance[6] equipped with a printer was used to weigh samples for quantitative TLC. A UV viewing box[7] and a tracing table[8] were used for observing and photographing TLC spots under UV irradiation or by transmission of white light. A calculator–printer[9] was used for statistical calculations. A 35-mm camera with black and white, color, and slide film was used[10].

Reagents, TLC Plates, and Pipets—Acetone and antimony trichloride were analytical reagent grade. Chloroform was NF grade. Bratton–Marshall reagent[11] was used as received. Absolute methanol was analyzed reagent grade. TLC plates[12] were precoated with silica gel.

Dexamethasone NF, betamethasone NF, and dexamethasone phosphate USP reference standards were used. Dexamethasone phosphate disodium salt was obtained as a research sample[13]. Micropipets[14] were used in TLC.

Preparation of 9-Fluoro-11β-hydroxy-16α-methylandrosta-1,4-diene-3,17-dione (IIα) and 9-Fluoro-11β-hydroxy-16β-methylandrosta-1,4-diene-3,17-dione (IIβ)—These compounds were synthesized for use as analytical standards by a modification (11) of the procedure of Rausser and Oliveto (16). The epimers were recrystallized twice to provide adequate purity. The α-epimer was recrystallized from acetone and hexane (16), and the β-epimer was recrystallized from methanol. The standards were dried in a drying apparatus at 55° and 5 mm and stored in a desiccator over phosphorus pentoxide overnight. Their purities were assessed from their melting points, UV and IR spectra, and TLC chromatograms.

TLC—All substances in methanol were spotted at origins 15 mm from the base of the plate. The chromatograms were developed with chloroform–acetone (9:1) until the solvent front ascended 170 mm above the origins. Equilibrated plate development was achieved in a Desaga gas-flushing tank fitted with stopcocks that allowed both addition of the mobile solvents and relief of any pressure imbalance arising from solvent mixing after the tank was covered. The spotted plates were developed three times before they were scanned with the densitometer. Plates used for quantitative determinations were scored 170 mm from the origin (15 mm from the top).

Sample Preparation and Procedure—Qualitative TLC was performed on the synthesized α- and β-epimers (IIα and IIβ), dexamethasone, betamethasone, dexamethasone acetate, dexamethasone phosphate, and dexamethasone phosphate disodium salt as well as on the epimeric impurity (IIα and IIβ) obtained from a contaminated commercial sample of the dexamethasone phosphate disodium injection solution. The densitogram of the epimeric impurity from the drug sample was compared with a chromatogram obtained from HPLC examination of another sample of the epimeric impurity extracted from the same batch of the commercial dexamethasone phosphate disodium solution for injection. The sample of the epimeric impurity and its high-performance liquid chromatogram were provided by the authors of the HPLC study (11). This qualitative work (11) included studies on the photographic methods of documentation conducted on concocted mixtures of the synthesized epimers (IIα and IIβ) and on the natural epimeric impurity (IIα and IIβ) found in a commercial injection solution of I.

Table I—Peak Heights of Epimeric 16-Methyl 17-Ketones in Synthetic Mixtures and Drug Impurity [a]

Sample	17-Ketone, %		Peak Heights[b]: $\frac{\alpha}{\alpha+\beta}$	CV
	α	β		
1	20	80	0.286	1.32[c]
2	30	70	0.371	1.10
3	40	60	0.451	0.95
4	50	50	0.527	0.68
5	60	40	0.597	0.70
6	70	30	0.677	0.78
7	Impurity		0.721	0.72
8	80	20	0.757	0.76

[a] Each plate was spotted once with each sample. [b] Average peak height fractions obtained from densitograms from 12 TLC plates. [c] Some plates failed to show appreciable resolution (Sample 1) because of nearness (1 cm) of the spot to the edge of the plate. Half of the plates gave resolution adequate to permit measurement.

The epimers (IIα and IIβ) were freshly synthesized for the quantitative study, and a sample of the drug impurity was freshly extracted. The prepared standard mixtures, containing various ratios of the synthesized α- and β-epimers, and a sample of the epimeric impurity extracted (11) from the commercial dexamethasone phosphate disodium injection solution were subjected to TLC. Quantitative data on the epimeric composition were collected by measuring the peak heights of the α- and β-epimers in densitograms of the standard 17-ketone mixtures and of the sample impurity.

For the quantitative study, six pairs of TLC plates were prepared. Eight spots of solutions were applied to each plate from a 1-μl pipet handled with a clamp. The spots were placed 25 mm apart, starting 10 mm from the left side of the plate. (The spots nearest the edges of the plate were used to evaluate the influence of "spot edge" proximity on densitometric measurements.) Each pair of plates was developed simultaneously in the same tank and subjected simultaneously to densitometry at a later date. In accordance with the data shown in Table I, the spots were numbered in increasing order from left to right; each spot contained either a mixture of the synthesized reference standards or the impurity from the drug sample.

Only one pair of plates was not from the same original manufacturer's packed carton. This pair and another pair were developed simultaneously three times in the same tank. Between the spotting, development, and densitometry steps, no special arrangements were made for storing the chromatographic plates other than keeping them in a closed cardboard box.

Stock solutions were prepared by individually dissolving 50.12 mg of α-epimer and 50.11 mg of β-epimer in 50.00 ml of absolute methanol. With these convenient concentrations (1.0 mg of 17-ketone/ml) and the use of volumetric pipets, mixtures of the synthetic 17-ketones with the percentages of α- and β-epimers designated in Table I were prepared. A freshly extracted sample of epimeric 17-ketones obtained from a previously described (11) sample of contaminated dexamethasone phosphate disodium was dissolved in methanol to give a concentration of ~1 mg/ml. The sample solutions were concentrated fivefold to provide concentrations within the range of those of the standard mixtures used to spot the TLC plates.

Treatment of Plates for Identification and Photography of Chromatographic Spots—Several methods were used to locate spots on the TLC plates. In the first method, the plates were viewed under shortwave UV irradiation, and the spots were located through fluorescence diminution of the fluorescent silica gel material. In the second method, the plates were sprayed heavily with a saturated solution of antimony trichloride in chloroform and then placed in an oven at 110° for 15 min. If the spots were not dark enough, the process was repeated. The spots also were observed by their fluorescence under longwave UV light. In a third procedure, the nonfluorescent plates were sprayed with a fluorescent material, Bratton–Marshall reagent, prepared by dissolving 200 mg of N-(1-naphthyl)ethylenediamine dihydrochloride in 10 ml of deionized water and adding 90 ml of methanol.

Black and white photographs of the fluorescent plates were taken at a view box under shortwave UV excitation. To photograph the colored spots obtained by treatment with antimony trichloride and heat, the plate was placed on the lighted surface of a tracing table[8] and photographed in color or black and white from above by transmitted light. The fluo-

[1] Model SD 3000, Schoeffel Instrument Corp., Westbrook, NJ 07675.
[2] Model ACTA MIV, Beckman Instruments, Fullerton, CA 92634.
[3] Model 337, Perkin-Elmer Corp., Norwalk, CT 06856.
[4] Carl Zeiss, New York, NY 10018.
[5] Kofler, Arthur H. Thomas Co., Philadelphia, PA 19105.
[6] Mettler balance model HL 52 and printer model GA 20, Mettler Instrument Corp., Hightstown, NJ 08520.
[7] Chromato-vue, model CC-20, Ultraviolet Products, San Gabriel, CA 91775.
[8] Model J-52 reader, Ionker, Gaithersburg, MD 20760.
[9] Model 9100A, Hewlett-Packard, St. Louis, MO 63144.
[10] Canon FTb 35-mm camera, Kodak Plus-X panchromatic, Kodacolor II, and Kodachrome 64 film.
[11] Certified ACS.
[12] Silica gel 60 F-254 and silica gel 60, E. Merck Laboratories, Elmsford, NY 10523.
[13] Merck Sharp and Dohme Research Laboratories, West Point, PA 19486.
[14] Drummond Scientific Co., Broomall, PA 19008.

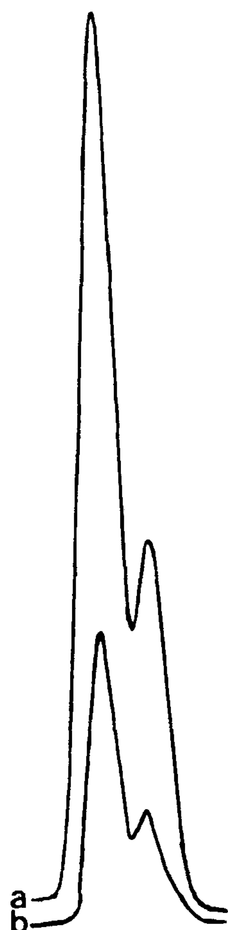

Figure 1—*Densitogram (a) and HPLC chromatogram (b) of drug impurity containing 16α- (left peak) and 16β-methyl (right peak) 17-ketones.*

rescence of these spots was also photographed by turning the TLC plate upside down and photographing the back (glass) side of the plate in a view box under longwave UV irradiation.

Spectrodensitometry—The operating conditions included a 150-w xenon lamp as the light source, an excitation wavelength of 240 nm, 2-mm band slit widths, a 3-mm plate beam width, a 10-mm plate beam length, measurement in reflectance and double-beam modes, a scanning speed of 1.27 cm/min, and a paper speed of 3.8 cm/min.

RESULTS AND DISCUSSION

Figure 1 shows a superimposition of the densitogram of the TLC separation (curve a) and the chromatogram of the HPLC separation (curve b); both demonstrate the separation of the α- and β-epimers of the impurity from the commercial drug injection. The TLC and HPLC estimations are in good agreement, and both indicate that the α- and β-epimers (IIα and IIβ) were ~3:1, respectively. Three developments of the TLC plate appeared to give the most satisfactory chromatograms.

The UV spectra of all materials were obtained just before qualitative chromatography, and no photodecomposition was detected by this method. The epimeric mixture from the drug impurity (IIα and IIβ), the synthesized α-epimer, and the synthesized β-epimer each showed maximum absorption at 240 nm, in agreement with reported values (11).

Table II gives R_f values obtained after one (R_f), two (R_{2f}), and three (R_{3f}) developments for densitometry. Table II includes data (under R_{2f}) on the use of nonfluorescent silica gel plates; these plates were sprayed with Bratton–Marshall reagent prior to viewing under shortwave UV light. Dexamethasone acetate was clearly detectable in the presence of dexamethasone or its epimeric 17-ketone oxidation products.

The system described here lends itself to the direct inspection of dexamethasone phosphate disodium drug samples and related samples for the presence of epimeric 16-methyl 17-ketones and possibly other oxidation products or impurities (Table II). To promote this objective, the TLC system retains dexamethasone 21-phosphate and dexamethasone 21-phosphate disodium at the origin, thus preventing interference with spots representing possible impurities being investigated, and distinguishes between dexamethasone 21-acetate, which is sometimes found at low levels in drug preparations of the respective parent corticosteroid (12), dexamethasone, and its 17-ketone oxidation products.

Table II—R_f **Values** [a] **of Substances Studied**

Substance	R_f [b]	R_{2f} [c]	R_{3f} [b]
16α-Methyl 17-ketone (IIα)	0.29	0.557	0.63
16β-Methyl 17-ketone (IIβ)	0.26	0.483	0.58
Dexamethasone	0.005	0.103	0.12
Betamethasone	0.005	0.097	0.11
Dexamethasone acetate	0.20	0.40	0.45
Dexamethasone 17-phosphate and 17-phosphate disodium	0.00	0.00	0.00

[a] The adsorbent was silica gel, and the solvent was chloroform–acetone (9:1). The R_f values are given for plates developed once (f), twice ($2f$), or three times ($3f$). [b] The R_f and R_{3f} data were collected from the same fluorescent silica gel plate under shortwave UV light; alternatively, the plate was treated with antimony trichloride followed by heat and observed under longwave UV light. [c] The silica gel plate was sprayed with Bratton–Marshall reagent and viewed under shortwave UV light; alternatively, the plate was treated with antimony trichloride followed by heat and observed under longwave UV light.

Furthermore, this method offers an R_f of about ~0.3 for the epimeric impurity; this R_f is ideal for multiple development of TLC plates because it allows maximum separation within the limit of two or three developments usually imposed by the time available to analyze one sample and by diffusion. Moreover, the Thoma (17) equation indicates that the ideal number of developments (N_{opt}) to obtain the maximum separation by unidimensional multiple chromatography of the two epimers with an average R_f value of 0.275 (R_f') is approximately three:

$$N_{opt} = \frac{-1}{\ln(1 - R_f')} = \frac{-1}{\ln 0.725} = 3.1 \quad \text{(Eq. 1)}$$

Theoretical consideration of the steric nature of the 17-ketone also suggests that adsorption occurs on the underside or α-side of 9-fluoro-11β-hydroxy-16-methylandrosta-1,4-diene-3,17-dione (II) where steric hindrance is minimal. In these terms, IIα would be adsorbed less firmly than IIβ, which is consistent with the observed, faster migration found for the α-epimer in the present TLC study and the previous HPLC study (11).

Vertical diffusion was diminished somewhat in the unidimensional multiple chromatography procedure in a fashion similar to that observed in multiple development or programmed multiple development; with the proper developing solvent, programmed multiple development causes more rapid migration in the lower areas of the spot, and this engenders longitudinal narrowing (18–20). Under the conditions investigated, more than three developments offered no improvement in separation. No attempt was made to examine centered programmed multiple development (18, 21) to improve the densitogram resolution.

Paper chromatographic liquid partition was described for identification of the epimeric corticosteroids dexamethasone (R_f 0.21) and betamethasone (R_f 0.16) by a 35-cm descending development with a mobile phase of chloroform saturated with formamide on filter paper[15] impregnated with 40% formamide in methanol (14). This method seemed to offer good prospects for separation of the epimers (IIα and IIβ), which differed from one another by configuration at the C-16 optical center in the exact same manner as the corticosteroids. Unfortunately the epimers followed the solvent front in this chromatographic system. A convenient TLC method to distinguish between dexamethasone and betamethasone would be to stir the material in question with sodium bismuthate in a suitable solvent, such as aqueous acetic acid, and to analyze a portion of the solution for 16α- or 16β-methyl 17-ketone by TLC with the use of reference materials.

In the quantitative studies, location of the spots on the plates was facilitated by placing the chromatograms under shortwave UV light. The spots then were subjected to densitometry in the reflectance mode with irradiation at 240 nm. Twelve pairs of resolved peaks (α- and β-epimers) from 12 plates were obtained for each standard mixture and sample, except for Standard Mixture 1 (20% α and 80% β). This mixture was spotted close (10 mm) to the edge of the plate, and occasional drift to the edge occurred, disrupting the chromatogram. In six cases where drift to the edge did not occur, excellent results were obtained and were included in the statistical calculations.

Small differences in the time chosen for spotting, development, and densitometry appeared to make little difference in the peak ratio data obtained. In one case, 3 days elapsed between spotting and development

[15] Whatman No. 1, Whatman Inc., Clifton, NJ 07014.

Table III—Treatment of Plates for Identification and Photography of Chromatographic Spots

Irradiation	Technique	Compound[a]	TLC Spot Colors on Prints: Color/Background[b] B and W Film	Color Film
	Untreated Fluorescent Plate			
Shortwave UV	Fluorescence extinction	Z	B/W	—
	Fluorescent Plate Treated with Antimony Trichloride and Heat			
White light	Transmitted and reflected light	D	B/W	YO/W
		B	B/W	YO/W
		α	B/W	YO/W
		β	B/W	YO/W
		DA	B/W	P/W
Longwave UV	Fluorescence	D	W/B	Y/B
		B	W/B	Y/B
		α	W/B	Y/B
		β	W/B	Y/B
		DA	W/B	R/B
	Nonfluorescent Plate Treated with N-1-(Naphthyl)ethylenediamine			
Shortwave UV	Fluorescence extinction	Z	B/W	—

[a] D = dexamethasone, B = betamethasone, α = IIα, β = IIβ, DA = dexamethasone acetate, and Z = all previously listed compounds. [b] B = black, W = white, Y = yellow, YO = yellowish orange, P = purple, and R = red.

of the plates, and 2 more days passed before densitometry was carried out; no significant difference in the results or peak ratios was observed. In fact, the plates may be stored for at least 2 months before densitometry. One plate was reexamined by densitometry 2 months later, and a comparison of data from both densitograms of this plate showed no significant difference. Another pair of plates taken from a different carton from the same manufacturer showed no difference in peak ratios when compared with the other plates.

The peak heights (represented as α and β in Table I) were measured and are presented as the ratio of the α-epimer to the sum of the α- plus β-epimers. Under ideal conditions, if this ratio is plotted against the percent of the α-epimer, a linear correlation should be obtained. The average ratio of each standard mixture and of the drug impurity is given in Table I. The peak heights obtained from the sample and from each standard mixture show good agreement and low coefficients of variation. A plot of the percent of the α-epimer *versus* the ratio of the α- and β-epimers placed all points practically on a straight line. A linear regression analysis of all 78 data from the standards gave $y = 0.770x + 0.139$, where y is the ratio defined earlier and x is the percent of the α-epimer, and a correlation coefficient (r) of 0.99925. From the peak height ratio of the impurity from the drug sample and the plot of the standard data, it was found that the impurity was composed of 75.2% α-epimer and 24.8% β-epimer. The peak of the β-epimer was broader and shorter than that of the α-epimer, and the β peak overlapped its neighboring α peak to a greater extent than the reverse effect. These factors give rise to the characteristics of the linear equation and the standard plot.

Stereoisomers often fail to separate chromatographically or may give incomplete resolution as was observed for the epimeric compounds in the present study. A peak ratio approach then may be desirable for quantitative studies, especially when the overlapping peaks are not symmetrical. In such an approach, the unknown sample is compared with a series of standard mixtures, each possessing a different ratio of the two contaminants. Groups of chromatograms must be examined; thus, a technique that offers simultaneous chromatographic determination is an asset. TLC provides an excellent means of collecting groups of data under similar conditions.

Operation of the spectrodensitometer was most efficient when the spots were applied 25 mm apart. By placing one spot 10 mm from the left edge and another 15 mm from the right edge, it was possible to evaluate whether the proximity of the spot to the edge of the plate influenced the analytical result. Spots placed 10 mm from the edge of the plate occasionally ran off the plate. Chromatograms not suffering from this disruption gave good results. However, only seven spots should be applied to a 20 × 20-cm plate.

The treatment of TLC plates for identification of the chromatographic spots is described in Table III. Fluorescent plates may be documented permanently by photography under shortwave UV irradiation in a view box.

Plates treated with antimony trichloride and heat gave yellow-orange spots for the epimers (IIα and IIβ), dexamethasone, and betamethasone but light-purple spots for dexamethasone acetate under white light. Distinct photographs showing black or colored spots on a white background were obtained by placing the TLC plates on a tracing table and photographing the transmitted white light.

Under longwave UV light, the spots obtained by treatment with antimony trichloride and heat fluoresced. In this case, the best results were obtained by photographing the plates upside down in the UV chamber; ideally, only the longwave exciting light will penetrate the glass, and only the colored fluorescent emitted light will transmit for the photographic record. The sorbent itself on the plate does not fluoresce. When these sprayed plates were viewed under longwave UV light, the epimers (IIα and IIβ), dexamethasone, and betamethasone appeared as yellow spots, whereas dexamethasone acetate appeared as a red spot.

With nonfluorescent plates, it is possible to apply a fluorescent material to the plate after development to observe the chromatogram by fluorescence diminution. This technique was effective when chromatograms of the epimers (IIα and IIβ) were sprayed with Bratton–Marshall reagent, N-(1-naphthyl)ethylenediamine, for subsequent viewing under shortwave UV light [Tables II (R_{2f}) and III].

Methods for positive and permanent documentation of laboratory results are important, particularly to regulatory bodies such as the Food and Drug Administration; photographic techniques were stressed in the present TLC study. Applied photographic documentation was discussed recently (15), and the general techniques were employed in this study.

The TLC system allows simultaneous detection of some related substances of interest in drug monitoring (Tables II and III), and densitometry is convenient for determining the relative amounts of 16α- and 16β-epimers of the 17-ketone impurity in dexamethasone-related drugs (Table I). There are distinct advantages in the use of TLC instead of HPLC for the analysis of 17-ketone oxidation products in dexamethasone phosphate disodium samples. When many samples must be examined for this contamination, a rapid qualitative technique such as TLC is ideal. The equipment is commonly available. With current techniques for multiple spotting and development, the method allows the simultaneous examination of numerous drug samples, a situation frequently encountered in drug-monitoring programs. The stability of the TLC chromatograms permits storage for reference, documentation by photography, and subsequent densitometry at the convenience of the analyst.

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Isolation and Identification of Impurities in 4-Acetyl-2-(2′-hydroxyethyl)-5,6-bis(4-chlorophenyl)-2*H*-pyridazin-3-one, an Antihypertensive Agent

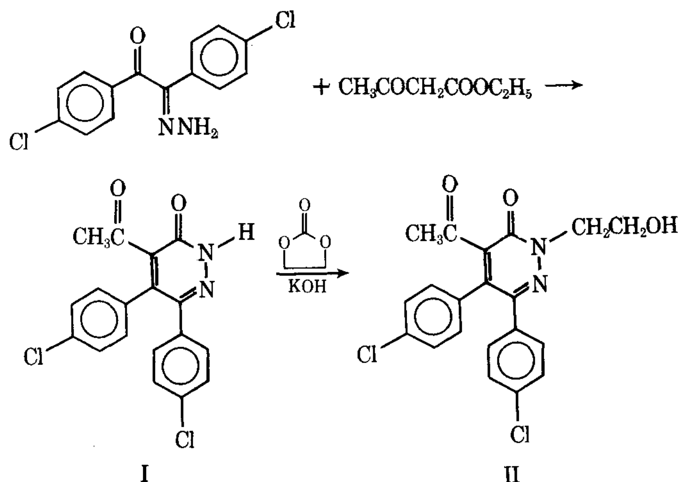
LARRY J. POWERS [x], DAVID J. ECKERT [*], and LANE GEHRLEIN [‡]

Received December 17, 1979, from the *T. R. Evans Research Center, Diamond Shamrock Corporation, Painesville, OH 44077.* Accepted for publication September 19, 1980. *Present address: Altex Scientific Inc., Berkeley, CA 94710. ‡Present address: Lederle Laboratories, Pearl River, NY 10965.

Abstract □ Three trace impurities found in 4-acetyl-2-(2′-hydroxyethyl)-5,6-bis(4-chlorophenyl)-2*H*-pyridazin-3-one (II), a novel antihypertensive agent, were isolated by a combination of low-pressure liquid chromatography and preparative TLC. These impurities were identified as the formate ester of II, a pyridazinone having a 2-methyl rather than the 2′-hydroxyethyl substituent, and a bis(pyridazinonyl)methane analog. In addition, the product of *O*-alkylation rather than of *N*-alkylation of 4-acetyl-5,6-bis(4-chlorophenyl)-2*H*-pyridazin-3-one (I) with ethylene carbonate was detected by high-performance liquid chromatography. The biological activity of these four impurities was compared to that of II.

Keyphrases □ 4-Acetyl-2-(2′-hydroxyethyl)-5,6-bis(4-chlorophenyl)-2*H*-pyridazin-3-one—synthesis, isolation of impurities by low-pressure liquid chromatography and preparative TLC and identification by high-pressure liquid chromatography, biological activity of impurities compared with parent compound □ High-pressure liquid chromatography—identification of impurities in 4-acetyl-2-(2′-hydroxyethyl)-5,6-bis(4-chlorophenyl)-2*H*-pyridazin-3-one, isolation of impurities by *low-pressure liquid chromatography and preparative TLC* □ Antihypertensive agents—4-acetyl-2-(2′-hydroxyethyl)-5,6-bis(4-chlorophenyl)-2*H*-pyridazin-3-one, isolation and identification of impurities, high-pressure liquid chromatography

The purification of a biologically active substance prior to pharmacological or toxicological evaluation is important in drug development. Such care is essential so that biological activity (including toxic effects) of trace impurities are not attributed to the molecule of interest. To ensure that trace impurities are innocuous, the biological activity of these substances can be studied independently if isolation, identification, and synthesis of the impurity can be successfully completed. The recent investigations of Reepmeyer and Kirchhoefer (1, 2) on the isolation and quantification of trace impurities in aspirin illustrate such an undertaking.



Scheme I

4-Acetyl-2-(2′-hydroxyethyl)-5,6-bis(4-chlorophenyl)-2*H*-pyridazin-3-one (II) is an antihypertensive agent prepared as illustrated in Scheme I. The various batches of II prepared in this manner have ~0.5% impurities after recrystallization. As part of a program (3, 4) to evaluate this compound, the major trace impurities present in the recrystallized material were isolated, identified, and synthesized. The antihypertensive activity and acute toxicity of four trace impurities were investigated.

EXPERIMENTAL[1]

Instruments—Melting points were determined on a capillary melting-point apparatus[2] and are uncorrected. NMR spectra were determined on a 90-MHz Fourier transform spectrometer[3] with tetramethylsilane as the internal reference. IR spectra were obtained on a grating spectrophotometer[4] as potassium bromide disks. Electron-impact mass spectra[5] were obtained by direct-sample inlet.

Chromatography—A low-pressure column was filled with dry silica gel (0.032–0.063 mm), and toluene–ethyl acetate–acetonitrile (2:1:1) (Solvent 1, Fig. 1) was pumped through the column until all air bubbles were removed. Column dimensions were 91 × 2.5 cm. Compound II (4.996 g) was dissolved in dimethylformamide (27 ml). Ten milliliters of this solution was injected onto the column, and the compound was eluted with Solvent 1. Fractions of 15 ml each were collected. The remainder of II was injected onto the column after the first injection had eluted. Fractions 11–28 and 74–84 (second injection) were combined to give A (Fig. 1).

A preparative thin-layer[6] plate (20 cm × 20 cm × 1.0 mm) was cleaned by eluting with acetone and then dried. Fraction A was dissolved in chloroform and applied to the preabsorbent. The plate was then eluted with Solvent 2. Subsequent preparative layer separations are summarized in Fig. 1.

High-performance liquid chromatography[7] (HPLC) with UV detec-

[1] Elemental analyses were performed on a Perkin-Elmer model 240 carbon, hydrogen, and nitrogen analyzer by the Central Analytical Department, Diamond Shamrock Corp.
[2] Thomas-Hoover, Arthur H. Thomas Co., Philadelphia, Pa.
[3] WH-90 Fourier transform NMR spectrometer, Bruker Instruments Inc., Billerica, Mass.
[4] Model 297, Perkin-Elmer Corp., Norwalk, Conn.
[5] Varian/MAT-CH-7 mass spectrometer with SS-166 data systems, Varian Associates, Palo Alto, Calif.
[6] SiO_2 PLQF 1000, Quantum Industries.
[7] Model 100 solvent delivery system (Altex Scientific, Berkeley, Calif.) with a model CV-6-UHPa-N60 sample injector (Valco Instrument Co., Houston, Tex.).

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