Stability Assays of Pharmaceutical Preparations by Quantitative Paper Chromatography

Steroid Hormones in Oil

By HENRY R. ROBERTS and KLAUS FLOREY

A quantitative paper chromatographic method has been developed which is routinely used as a stability assay for steroid esters having a 4-dehydro-3-keto configuration. The method involves: (*a*) a separation between the parent steroid ester and the free steroid, (*b*) the quantitative elution of the steroid ester from the chromatogram using an acidified methanolic solution of isonicotinic acid hydrazide, and (*c*) a spectrophotometric analysis of the eluate. Recoveries in excess of 95% are obtained by simultaneously chromatographing replicates of standard and sample solutions on the same chromatogram. A detailed account of the procedure is presented using, as examples, the assay of 17-α-hydroxyprogesterone caproate and testosterone enanthate in oil formulations.

WHEN DEVELOPING analytical procedures for the determination of active ingredients in pharmaceutical preparations, it is necessary to make a distinction between content and stability assays. For a content assay, any characteristic property of the molecule amenable to convenient quantitation can be used. A stability assay, on the other hand, has to show whether or not the amount originally put into a formulation has undergone changes which, with time, will alter its pharmacological potency. A stability assay must, therefore, measure either the intact active ingredient or its breakdown products.

In cases where breakdown products are either difficult to measure chemically because they closely resemble the active ingredient or are altogether unknown, we have found it convenient to follow the stability of formulations by measuring the active ingredients by quantitative paper chromatography. In quite a number of cases, this method reduces the need for elaborate extraction procedures, especially when two or more similar components have to be separated. It also eliminates the cumbersome task of preparing, for comparison, blank formulations which contain all the ingredients except the active one.

Previous workers (1–3) have described the application of quantitative paper chromatography for the assay of various pharmaceuticals. We have adapted quantitative paper chromatography specifically to measure product stability of a great variety of pharmaceutical preparations. In this paper we wish to report on techniques which permit the quantitation of 4-dehydro-3-keto steroids with isonicotinic acid hydrazide (4). The examples chosen are the quantitative determination of two steroid hormones, testosterone enanthate and 17α-hydroxyprogesterone caproate in oil formulations.

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EXPERIMENTAL

The paper chromatographic procedure involves, essentially, two steps: (*a*) the separation of the active component from its hydrolytic product and (*b*) the elution of the parent compound from the chromatogram followed by a quantitative spectrophotometric analysis of the eluate.

Preparation of Standards.—The reference standard solution should contain 1 mg. of the steroid hormone per ml. of solution. In a tared volumetric flask, the appropriate amount of steroid is dissolved in the smallest volume of N,N-dimethylformamide. Then methyl isobutyl ketone is added to the mark. The reference standard material should be from the same lot as the formulation undergoing analysis.

Preparation of Sample.—The amount of sample which will yield, based on theory, 1 mg. of the hormone per ml. of solution is weighed into a tared volumetric flask, dissolved, and diluted to the mark with methyl isobutyl ketone.

Filter Paper.—Whatman No. 1, $5^3/_4$ in. × 18 in., shape ERS, with 5 cut-outs. Prior to spotting, the papers are immersed for a minimum of 30 minutes in each of two 95% ethanol washes in order to minimize the concentration of ultraviolet light absorbing impurities in the paper. A large Pyrex baking dish can be used satisfactorily to wash 20 folded sheets at one time. The strips are folded but not creased. Following the second alcohol wash, the strips are air dried.

Solder is used to mark the strips with two lines prior to the spotting step. The origin line is $3^1/_4$ in. from the top.

The second line, $2^1/_4$ inches from the top, serves as a folding guide, providing the strip with a $2^1/_4$-inch tab which is inserted into the solvent trough.

Developing Solvent.—Methylcyclohexane saturated with diethylene glycol monoethyl ether[1] (DGME) (5).

[1] 2-(2-Ethoxyethoxy)ethanol, marketed as a Carbitol by Carbide and Carbon Chemical Co.

Chromatographic Chamber.—Any conventional chamber used for descending paper chromatography is suitable.

Eluting Solution.—A 1-Gm. quantity of isonicotinic acid hydrazide is dissolved in 1 L. of absolute methyl alcohol containing 1.25 ml. of concentrated hydrochloric acid.

Paper Chromatographic Procedure.—Two chromatograms are run per sample. The filter paper contains six $^3/_4$-inch strips which are spotted as illustrated in Fig. 1.

Blow-out pipets, 0.1 ml. capacity, graduated in 0.01 ml., are used to apply the standards and the sample to the paper. The solutions are applied in aliquots of approximately 0.005 ml. so that the area of application remains small. A stream of warm air is directed on the spot after each aliquot is applied. The air is warmed by passing it through heated coiled copper tubing.

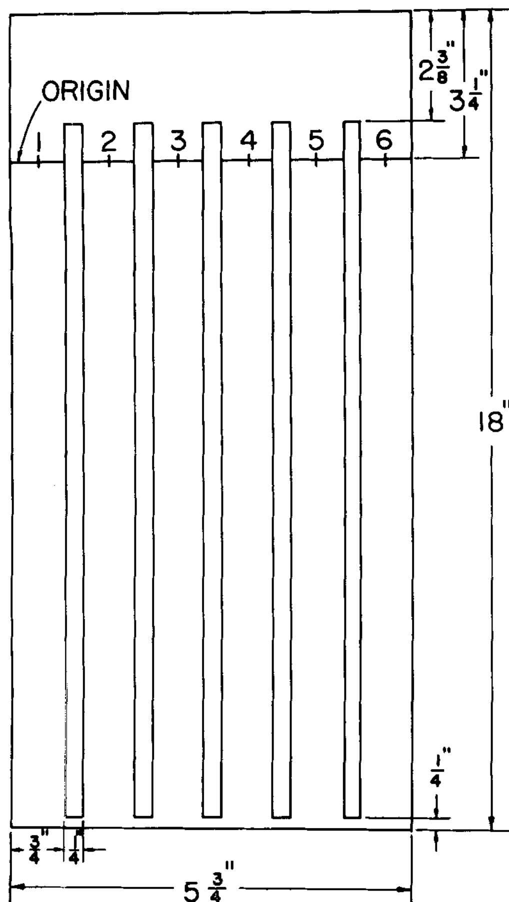

Fig. 1.—Diagram of spotting arrangement for assay of sex hormones in parenteral solutions.

	No. 1 Chromatogram	No. 2 Chromatogram
1 =	Standard, 0.05 ml.	Standard, 0.05 ml.
2 =	Standard, 0.10 ml.	Standard, 0.10 ml.
3 =	Sample, 0.05 ml.	Sample, 0.10 ml.
4 =	Sample, 0.05 ml.	Sample, 0.10 ml.
5 =	Sample, 0.05 ml.	Sample, 0.10 ml.
6 =	Paper blank	Paper blank

Table I.—Paper Chromatographic Conditions for Assay of Sex Hormones[a]

Compound	Time, hr.
Testosterone enanthate	$2^1/_2$
17α-Hydroxyprogesterone caproate	4

[a] Paper impregnating solution, 30% DGME in chloroform; developing solvent, methylcyclohexane saturated with DGME.

A spotting board, Fig. 2,[2] while not absolutely essential, offers a distinct advantage. It not only provides a support for the paper but it also holds the paper, by vacuum, in a fixed position. This permits spotting with one hand while the other hand is free to dry the spot after each application.

The spotted strips are impregnated with DGME by dipping them into the appropriate solution of DGME in chloroform, see Table I. The short end of the strip ($3^1/_4$ inches from the origin) is dipped first, just up to the origin, and blotted lightly. The remainder of the strip is then passed through the impregnating solution with a rapid pass over the origin. In this manner, the entire strip, including the spotted origin, becomes impregnated with DGME. Since the extent of DGME impregnation will influence the mobility of the steroid being assayed, it is important that the DGME in chloroform solution be prepared accurately. Also, since evaporation of chloroform will alter the concentration of DGME, it is best if approximately 100 ml. of dipping solution is used to impregnate two to three chromatograms.

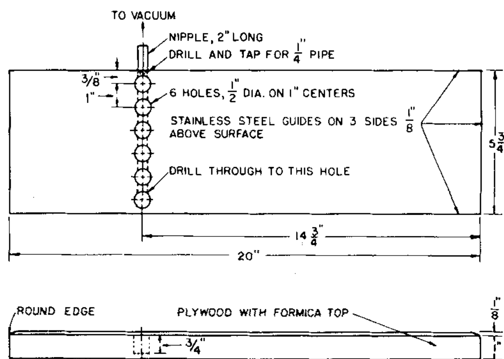

Fig. 2.—Board for spotting filter paper strips.

The impregnated strips are hung in a hood until the odor of chloroform can no longer be detected, and then they are placed in the chamber, the bottom of which is covered with DGME saturated with methylcyclohexane. The tab ends of the strips are placed into the troughs which contain the developing solvent, methylcyclohexane saturated with DGME. Following solvent development, Table I, the strips are removed from the chamber and dried in a mechanical convection oven for 20 minutes at 90°.

The positions of the steroids on the chromatograms are located by examining the strips through a fluorescent paddle over an ultraviolet light source.[3]

[2] The board shown here is a modification of the original design for which we are indebted to John M. West of these laboratories.

[3] The simple and efficient U.V. light source and paddle described here are a modification of the original design of Drake, *et al.* (6). We are indebted to R. W. Thoma and J. R. Gerke of these laboratories for these modifications.

The locations of the steroids are seen as black areas since they absorb the U.V. radiation and block the fluorescence. The source of ultraviolet radiation is a wooden box (8 in. × 8 in. × 21 in.) containing a 15-watt germicidal lamp (General Electric, 17 inch, No. G 1578) in the bottom and covered on top with three $6^1/_2$ in. sq. pieces of red-purple Corex A filters (No. 9863, Corning Glass Works, Corning, N. Y.).

The paddle is made from $^1/_8$ in. clear plexiglas and measures 7 in. square with a $1^1/_2 \times 4$ in. handle. It is coated with a thin layer of green fluorescing zinc silicate as follows: 6 ml. of collodion is measured into a 50-ml. glass-stoppered graduated cylinder and diluted to 22 ml. with acetone. To this is added 2 Gm. of zinc silicate phosphor (No. 609, E. I. DuPont de Nemours and Co., Towanda, Pa.). The mixture is shaken thoroughly and poured onto the paddle. The paddle is tipped in all directions so that the whole surface is evenly covered with a thin layer. Excess material is dripped off the paddle in the process of distributing it evenly.

The areas of the strips containing the steroids are marked with a solder pencil, cut out and folded, and placed in 50-ml. Erlenmeyer flasks. A paper blank, equal in area to the standard and sample segments, is included for each chromatogram (see Fig. 1). A piece of plywood mounted with 48 flask clamps (No. XCOO50, New Brunswick Scientific Co., Inc., New Brunswick, N. J.) holds the flasks during the elution step. A 10-ml. quantity of the isonicotinic acid hydrazide solution is added to each flask, the flasks covered with aluminum foil to prevent the evaporation of the methanol, and then shaken for 60 minutes on a reciprocating shaker (130 strokes per min.). A variable reciprocating laboratory shaker manufactured by Eberbach (No. G-21827, Emil Greiner Co.) is ideal for this step. The 60-minute shaking period is necessary for (*a*) the elution of the steroid and (*b*) the reaction, at room temperature (*ca.* 22°), between the eluted 4-dehydro-3-keto steroid and the isonicotinic acid hydrazide reagent to form a yellow hydrazone.

The solution is then decanted into a 1-cm. Corex cell and the absorbance is read at 415 mμ, using a Beckman DU spectrophotometer. Absolute methanol is used as the reference solution. The standard and sample readings are corrected for cell readings and for the paper blank.

Calculations.—The absorbance readings are recorded on a data sheet which contains all the information necessary to calculate the concentration of steroid in the sample. Table II gives the data obtained in a typical analysis.

For each of the two chromatograms, absorbance readings are obtained for the standard at two levels and for the sample at one level, in triplicate. The absorbance readings of the two standards for each of the chromatograms are converted to the equivalent absorbance for the 0.1 ml. standard concentration and averaged. The three absorbance readings of the sample are converted to 0.1 ml. readings and averaged.

The concentration of the steroid in the sample is calculated from the following equation

$$\text{mg. of steroid per ml. formulation} = \frac{A \times B \times C \times D}{E \times F \times G}$$

where A = average absorbance of 10 ml. eluate of 0.1 ml. chromatographed sample, B = weight in mg. of 0.1 ml. chromatographed standard, C = volume in ml. of sample dilution, D = specific gravity of formulation, E = average absorbance of 10 ml. eluate of 0.1 ml. chromatographed standard, F = weight in Gm. of sample, and G = volume (0.1 ml.) of chromatographed sample.

The final concentration of the steroid in the sample is obtained by averaging the values obtained for each of the two chromatograms.

Sample Calculation.—Table II, chromatogram No. 1

$$\text{mg. testosterone enanthate per ml.} = \frac{0.119 \times 0.1048 \times 100 \times 1.0127}{0.180 \times 0.1752 \times 0.1} = 400$$

RESULTS AND DISCUSSION

Recoveries in excess of 95% must be obtained if any analytical method is to serve as an effective stability assay. The accuracy and precision of the method were established by determining the 17α-hydroxyprogesterone caproate content of a Delalutin 2X preparation. Ten aliquots were weighed and diluted; two chromatograms were run per aliquot. The average value of 253 mg./ml. obtained from 10 individual assays (Table III) differed from the theoretical value by 1.2%. In every instance, no individual assay differed from the theoretical value by more than 3.2%, well below the 5% maximum limit placed on the assay. The standard deviation, 3.74 mg./ml., indicates that the assay can be repeated with good precision.

Accuracies of this magnitude are obtained since the standards and sample are run on the same chromatogram in replicate and both are exposed simultaneously to the same chromatographic and assay conditions. Table IV contains additional data indicating that assays with recoveries in excess of 95% are obtained consistantly.

The hormone preparations described here are dissolved in sesame or castor oil. They contain, in addition, some combination of benzyl alcohol, benzyl benzoate, and chlorobutanol.

Not only does the paper chromatographic procedure separate the active ingredient from its hydrolytic product but it achieves this without the need of any elaborate extraction procedure. A simple dilution prior to spotting is all the sample preparation required.

A marked separation is achieved on the chromatogram between the parent steroid esters and the free steroids. Testosterone and 17α-hydroxyprogesterone remain near the origin while the esters migrate down the strip. While the intact ester is measured, the procedure also permits a visual identification and, if necessary, quantitative measurement of the hydrolytic product. However, the stabilities of the steroid esters in the oil formulations described here are excellent and no breakdowns have been observed upon prolonged storage at elevated temperatures, Table V.

The other components in the formulation do not interfere. The only possible interference originated with the benzyl benzoate. This compound is a U.V. absorber which has a chromatographic mobility similar to the steroids being assayed. While a clear

TABLE II.—DATA SHEET FOR ASSAY OF TESTOSTERONE ENANTHATE IN DELATESTRYL 2X

Project:	Te.204-53 Delatestryl 2X	Filter Paper:	EtOH Washed Finger Strips	Wavelength:	415 mμ
Sample:	Stability -50°C, 1 month	Developing Solv.:	Methylcyclohexane saturated with Carbitol	Slit Width:	0.06
Assay:	Testosterone Enanthate	Developing Time:	2-1/2 hrs.	Cell Corrections:	
Sample Wt.:	175.2 mg./100 ml.	Drying Cond.:	20 min. 90°C.	No.1:	+0.001
Sp. Gr. Sa.:	1.0127	Eluting Solution:	10 ml. Hydrazid	No.2:	+0.001
Wt. Standard:	26.2 mg./25 ml.	Eluting Time:	60 Min.	No.3:	+0.002
				Corr. Solvent:	Abs. Methanol

Chrom.	Flask No.	Cell No.	Spot	O.D.	Corrected O. D.		O.D. St.=0.10 ml. Sa.=0.10 ml.	O.D. (Av.) St.=104.8γ Sa.=0.10ml.	Conc. mg./ml. Formulation
					Cell	Blank			
1	1	1	Std.=0.05 ml.	0.142	0.141	0.089	0.178	0.180	
	2	2	Std.=0.10 ml.	0.234	0.233	0.181	0.181		
	3	3	Sa.=0.05 ml.	0.113	0.111	0.059	0.118		
	4	1	Sa.=0.05 ml.	0.112	0.111	0.059	0.118	0.119	400
	5	2	Sa.=0.05 ml.	0.113	0.112	0.060	0.120		
	6	3	Paper Blank	0.054	0.052	-			
2	7	1	Std.=0.05 ml.	0.143	0.142	0.088	0.176	0.178	
	8	2	Std.=0.10 ml.	0.236	0.235	0.181	0.181		
	9	3	Sa.=0.10 ml.	0.169	0.167	0.113	0.113		
	10	1	Sa.=0.10 ml.	0.169	0.168	0.114	0.114	0.115	392
	11	2	Sa.=0.10 ml.	0.173	0.172	0.118	0.118		
	12	3	Paper Blank	0.056	0.054				

Average: 396 mg./ml.

separation between the steroid and benzyl benzoate is not obtained, its presence is negated by reacting the steroid with isonicotinic acid hydrazide.

The use of acidified alcoholic isonicotinic acid hydrazide solution as a colorimetric reagent and as a paper chromatographic spray reagent for the detection of 4-dehydro-3-keto steroids have been described by a number of investigators (4, 7, 8). The manner in which it is utilized as a simultaneous eluant and colorimetric reagent as reported here has not, to the authors' knowledge, been described previously.

The absorption spectra of the isonicotinyl hydrazones of testosterone enanthate and 17α-hydroxyprogesterone caproate are similar to the spectrum given by Umberger (4) for the hydrazone of testosterone with a maximum at 380 mμ. The absorbances of the eluates can be measured in two different ways, both giving equally reliable results (Table VI). Absorbances of the eluates can be read at 380 mμ using the hydrazide reagent as a reference solution. At this wavelength the paper blank readings are extremely low. However, due to evaporation of the solvent, the concentration of the reference solution changes very rapidly and has to be replaced after every three readings. This becomes tedious and time consuming when the absorbances of a large number of eluates have to be read. There-

TABLE III.—ACCURACY AND PRECISION OF THE PAPER CHROMATOGRAPHIC DETERMINATION OF 17-α-HYDROXYPROGESTERONE CAPROATE IN DELALUTIN 2X

Aliquot No.	Found, mg./ml.	Deviation from Theory,[a] %
1	254	+1.6
2	258	+3.2
3	249	−0.4
4	258	+3.2
5	248	−0.8
6	254	+1.6
7	249	−0.4
8	251	+0.4
9	256	+2.4
10	251	+0.4
Average	253	+1.2
Standard deviation	3.74	
Coefficient of variation, %	1.48	

[a] Theoretical value, 250 mg./ml.

TABLE IV.—PAPER CHROMATOGRAPHIC ANALYSES OF SEX HORMONES IN PARENTERAL DELAHORMONE PREPARATIONS

Active Ingredients per ml.	Found, mg./ml.	Deviation From Theory, %
Testosterone enanthate,[a] 200 mg.	200	0
	206	+3.0
	198	−1.0
	196	−2.0
Testosterone enanthate,[b] 400 mg.	407	+1.8
	410	+2.5
	405	+1.2
	403	+0.8
17α-Hydroxyprogesterone caproate,[c] 125 mg.	120	−4.0
	126	+0.8
17α-Hydroxyprogesterone caproate,[d] 250 mg.	255	+2.0
	257	+2.8
	256	+2.4
	255	+2.0
Testosterone enanthate 180 mg.;	178	−1.1
Estradiol valerate,[e, g] 8 mg.	181	+0.5
17α-Hydroxyprogesterone caproate 250 mg.;	254	+1.6
Estradiol valerate,[f, g] 5 mg.	250	0
	252	+0.8

Squibb trade names: [a] Delatestryl. [b] Delatestryl 2X. [c] Delalutin. [d] Delalutin 2X. [e] Deladumone 2X. [f] Deluteval 2X. [g] Quantitative paper chromatographic assay of estrogens described in paper II of this series.

TABLE V.—PAPER CHROMATOGRAPHIC ANALYSES OF SEX HORMONES IN OIL UNDERGOING STABILITY STUDIES

Sample No.	Active Ingredient per ml.	Time, Mo.	Temperature, °C. 5	22	40	50
			Found, mg./ml.			
1	Testosterone enanthate, 180 mg.	12	...	181	187	185
2	Testosterone enanthate, 200 mg.	12	211	214	214	209
3	Testosterone enanthate, 200 mg.	12	206	196	205	199
4	Testosterone enanthate, 200 mg.	24	...	195	209	195
5	17α-Hydroxyprogesterone caproate, 125 mg.	12	126	125	132	129
6	17α-Hydroxyprogesterone caproate, 125 mg.	12	126	127	128	132
7	17α-Hydroxyprogesterone caproate, 125 mg.	18	...	126	128	129
8	17α-Hydroxyprogesterone caproate, 250 mg.	6	258	253	257	254
9	17α-Hydroxyprogesterone caproate, 250 mg.	6	...	257	256	251
10	17α-Hydroxyprogesterone caproate, 250 mg.	12	255	257	256	255

TABLE VI.—COMPARISON OF TESTOSTERONE ENANTHATE CONTENT CALCULATED FROM ELUATE ABSORBANCES

	380 mμ[a]		415 mμ[b]	
Chromatogram	Found, mg./ml.	Deviation from Theory,[c] %	Found, mg./ml.	Deviation from Theory, %
1	394	−1.5	406	+1.5
2	403	+0.75	396	−1.0
3	416	+4.0	410	+2.5
4	389	−2.75	405	+1.25
5	416	+4.0	414	+3.5
Average	404	+1.0	406	+1.5

[a] Read against the isonicotinic acid hydrazide reagent; fresh reagent blank for every three eluate readings. [b] Read against absolute methanol; same blank for all eluate readings. [c] Theoretical value, 400 mg./ml.

fore, as described in the experimental part, the absorbances of the eluates are read at 415 mμ against methanol as the reference solution. At this wavelength, lower paper blank readings ($A \backsim 0.050$) are obtained than at 380 mμ ($A \backsim 0.200$). The high paper blank at 380 mμ is primarily due to the absorbance of excess hydrazide in the eluates (4).

The generally accepted procedure when working with Zaffaroni-type chromatographic systems is to impregnate the paper with the stationary phase prior to spotting the steroidal solution. For quantitation, spotting the paper prior to impregnation is more expeditious since the spotting-dipping sequence provides for better control over the uniformity of the stationary phase. Furthermore, papers can be spotted well in advance of chromatography. No distortion or diffusion of the zones has been noted when the impregnation of the spotted origin is done rapidly, as described.

The use of slotted sheets of filter paper offers a number of apparent advantages. The steroids corresponding to the standard and sample applications are confined in their solvent migration to their own $^3/_4$-in. wide strips. Lateral diffusion is suppressed and contamination from adjacent spots can never occur. Also, the area of each of the strips containing the steroid, as well as the paper blank segment, are at all times equal since only the north-south boundaries have to be ascertained.

The design of the fluorescent screen as a paddle makes for easy marking of the steroid segments. The edges of the paddle are used as guides while, at the same time, assuring that all the absorption is confined to the outlined zones.

SUMMARY

1. A quantitative paper chromatographic assay has been developed for the assay of 4-dehydro-3-keto steroids using an acidified methanolic solution of isonicotinic acid hydrazide.

2. The procedure is used routinely to measure the stability of a number of sex hormones in oil preparations. The assay of 17α-hydroxyprogesterone caproate and testosterone enanthate are described in detail.

3. The method is simple and accurate and permits the simultaneous visual identification and, if necessary, quantitative measurement of any hydrolytic product.

4. No extraction of the sex hormone is required. A simple dilution prior to chromatography is the only sample preparation required.

5. Accuracies in excess of 95% are obtained by simultaneously chromatographing replicates of standard and sample solutions on the same chromatogram.

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Preparation and Pharmacological Properties of Some Benzyl Derivatives of Diphenylacetic Acid

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Benzyl derivatives of diphenylacetic acid were prepared from the starting material 2-chlorodiphenylacetyl chloride. The compounds synthesized were the N-benzyl derivatives of 2-chloro-, 2-hydroxy-, 2-acetoxy-, 2-dimethylaminoethoxy-, and 2-benzylaminodiphenylacetamide; N-benzyl-N-methyl derivatives of 2-chloro- and 2-hydroxy-diphenylacetamide; O-benzyl derivatives of 2-chloro- and 2-hydroxy-diphenylacetamide; and the compound 2-benzylaminodiphenylacetamide. The compounds have limited aqueous solubility so the full extent of the possible pharmacologic actions could not be studied. These agents orally in doses of 1 Gm./Kg. produced no toxic symptoms but the N-benzyl-N-methyl-2-chloro- and the N-benzyl-2-hydroxy-diphenylacetamides inhibited the passage of a charcoal meal, indicating antispasmodic activity similar in potency to that of atropine. Intravenously, the compounds were without cardiovascular or respiratory effects. Intraperitoneally, N-benzyl-2-hydroxy and N-benzyl-2-acetoxy diphenylacetamides showed antipentamethylene tetrazol activity, and one of these also abolished the convulsive actions of electrical shock.

VARIOUS diphenylacetic acid derivatives have long been of interest as antispasmodic agents. The relatively recent literature indicates that certain compounds containing the diphenylacetyl moiety have also been found to possess sedative or anticonvulsant properties. For example, Billman, *et al.* (1, 2), have studied a series of substituted derivatives of 2-aminodiphenylacetamide possessing both anticonvulsant and antispasmodic activity. Among references in the patent literature, N-acetoxy (diphenylacetyl)acetamide (3) is claimed to be an antiepileptic without untoward effects and having extremely low toxicity in animals.

Also apparent from the recent literature is the frequency with which the benzyl group is found, usually as an amide or ester, in compounds with notable properties as sedatives or anticonvulsants. Kushner, *et al.* (4), also observed that many compounds containing a benzylamide moiety possessed pronounced anticonvulsant activity and their study of a variety of such substances resulted in the development of N-benzyl-β-chloropropionamide. N-Benzylmandelamide has

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