A decreased particle size in these drug–excipient systems was confirmed by differential thermal analysis data; in most cases, a 2–3° depression in the melting point was observed. Hydrogen bonding in a gel matrix was apparently responsible for the formation of a metastable configuration in two systems: indomethacin and probucol. The polymorphic forms were first detected by differential thermal analysis and confirmed by X-ray and IR data. The existence of multiphase solids was a consequence of the presence of fumed silicon dioxide acting as a nucleating agent for the metastable crystals. Although the dissolution rates of the isolated polymorphs were found to be identical to those of the original compounds, it is quite possible that in the dispersed state the metastable form dissolved at a rate faster than the stable form.

Since drug–excipient interactions do readily occur in normal manufacturing procedures, and since these interactions not only alter the physical and chemical properties of the drug but also appreciably affect the physiological availability of the drug from the dosage form, it is highly desirable that such interactions be considered as a part of strict in-process quality control.

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▲ To whom inquiries should be directed.

GLC Determination of Chlormadinone Acetate in Plasma

R. J. BOPP▲, H. W. MURPHY, J. F. NASH, and C. R. NOVOTNY

Abstract □ Plasma samples obtained from monkeys after oral administration of 0.5–2.0 mg. of chlormadinone acetate were extracted with hexane. The hexane extracts were washed, concentrated, and analyzed by GLC using a ^{63}Ni electron-capture detector. The analyses were performed on a 30.5-cm. (1-ft.) column packed with either 3.8% methyl silicone gum rubber on silanized diatomite or a mixed phase consisting of 3.0% methyl silicone gum rubber and 1.5% polyethylene glycol 20,000 on silanized diatomite. Levels as low as 1.0 ng. steroid/ml. plasma were detected by this procedure when 5-ml. samples of plasma were available.

Keyphrases □ Chlormadinone acetate—GLC analysis, plasma, monkeys □ GLC, electron capture—analysis, chlormadinone acetate in plasma, monkeys

Since the introduction of sequential oral contraceptives in the spring of 1965, chlormadinone acetate, 6-chloro-17-hydroxypregna-4,6-diene-3,20-dione acetate (I), has been used as a progestogen in one of these drug products (1). In 1967, Martinez-Manautou *et al.* (2) reported on the contraceptive efficacy of 0.5-mg. daily oral administration of chlormadinone acetate as a low-dose progestogen, free of estrogen. They considered the 0.5-mg. tablet an effective, remarkably benign oral contraceptive. During 1968, the 0.5-mg. tablet of chlormadinone acetate was introduced to the market in Mexico, France, and Argentina.

To demonstrate biological availability of chlormadinone acetate from tablets, it became necessary to develop a sensitive analytical method for the detection and quantitation of the drug in plasma. Work by Koons and Scroggs (3) and Donoho *et al.* (4), describing the determination of chlormadinone acetate in animal feeds and cattle tissue, was helpful in the development of such a method. The method described in these studies is based upon GLC using electron-capture detection. The initial studies were performed in monkeys using 0.5-, 1.0-, and 2.0-mg. doses of chlormadinone acetate. This method recently proved satisfactory for the monitoring of blood levels of this drug in humans (5).

EXPERIMENTAL

Reagents—Chlormadinone acetate, chlormadinone-C_1-^3H acetate, and chlormadinone caproate (II) were used[1]. Hexane and benzene were spectral quality while toluene and methanol were

[1] Supplied by Syntex, S. A., Mexico D. F., Mexico.

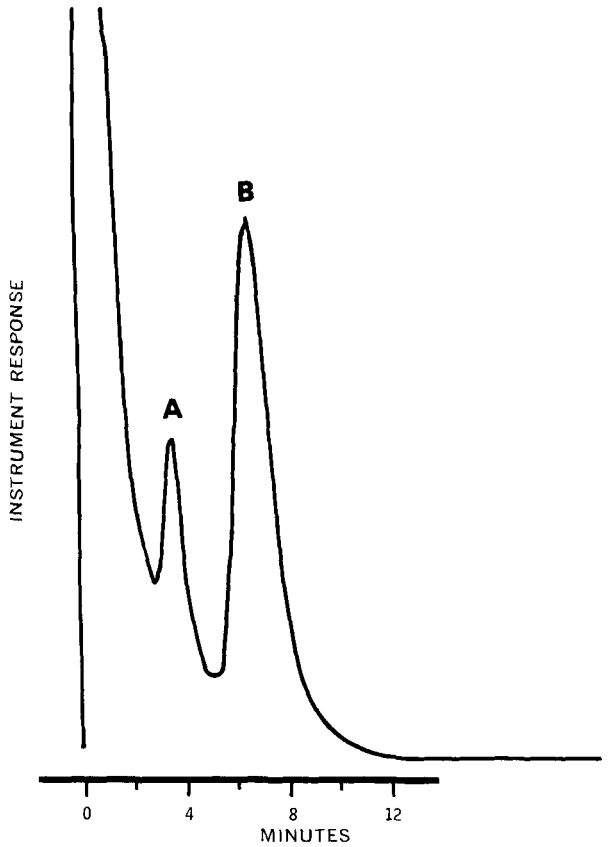


Figure 1—*Gas chromatogram of an extract of 5 ml. of human plasma to which 25 ng. of chlormadinone acetate (A) and 125 ng. of chlormadinone caproate (B) were added.*

analytical reagent quality. The following were also used: dimethyldichlorosilane[2], methyl silicone oil (SE-30)[2], Carbowax 20M[2], Diatoport S (80–100 mesh)[2], and methyl vinyl silicone gum rubber (UC W-98)[3]. A molecular sieve, 5A (80–90 mesh), was obtained from a commercial source[4].

A standard solution of chlormadinone acetate was prepared by dissolving 1.0 mg. of the compound in 100 ml. of anhydrous methanol. One milliliter of the methanolic solution was diluted to 200 ml. with deionized water to yield a concentration of 50 ng. chlormadinone acetate/ml.

A standard solution of chlormadinone caproate was prepared by dissolving 2.5 mg. of the compound in 100 ml. of hexane. One milliliter of the hexane solution was then diluted to 100 ml. with the same solvent to yield a concentration of 250 ng. of chlormadinone caproate/ml. This compound served as the mass internal standard.

Apparatus—GLC was accomplished using a chromatograph[5] equipped with a nickel (^{63}Ni) electron-capture detector. Samples were taken to dryness under nitrogen by use of an evaporator[6]. Dissolution of the dried samples in benzene was aided by the use of ultrasonic vibration[7]. Fifteen- and 20-ml. tapered glass centrifuge tubes[8] with Teflon-lined screw-caps were used.

Chromatographic Conditions—A recorder[9] (−0.2–1.0 mv.) was operated at a chart speed of 0.63 cm. (0.25 in.)/min.

The carrier gas, 10% methane–90% argon (Matheson), was used at a flow rate of 250 ml./min. The carrier gas was filtered through a 1.83-m. × 0.63-cm. (6-ft. × 0.25-in.) copper tubing filled with 80–90-mesh molecular sieve 5A. The filter was preconditioned overnight at 200° while passing through dry nitrogen.

[2] Applied Science Laboratories, State College, Pa.
[3] F and M Scientific Co., Indianapolis, Ind.
[4] Analabs, Inc., Hamden, Conn.
[5] F and M, model 402, Hewlett Packard, Skokie, Ill.
[6] N-Evap, Organomation Associate, Shrewsbury, Mass.
[7] Sonogen, Cole-Parmer, Chicago, Ill.
[8] Matheson Scientific Co., Chicago, Ill.
[9] Honeywell Elektronik 16.

Table I—Typical Data Obtained for a Calibration Curve of Chlormadinone Acetate

Weight (ng.) of Chlormadinone Acetate/ml.	Peak Height Ratio
1	0.065
2	0.140
3	0.195
4	0.265
5[a]	0.365

[a] The actual chromatogram from which this ratio was obtained is given in Fig. 1.

A 30.5-cm. by 3.0-mm. i.d. (1-ft. by 0.13-in. i.d.) all-glass column packed with either 3.8% W-98 on Diatoport S or a mixed phase consisting of 3.0% SE-30 and 1.5% Carbowax 20M on Diatoport S and operated at temperatures of 215 and 230°, respectively, was used in the analysis. The glass column and glass wool used in preparing the column were siliconized prior to use (6).

The 2.54 × 0.31-cm. (1 × 0.125-in.) metal tubing leading from the end of the column to the detector was heated to 240°. The detector itself was at a temperature of 240°. Under these operating conditions, chlormadinone acetate had a retention time of 3.6 min. and chlormadinone caproate had a retention time of 6.6 min. (Fig. 1). With the electron-capture detector operating at a pulse interval of 150 μsec., 6 ng. of chlormadinone acetate gave full-scale deflection at range 10, attenuation 2 (8×10^{-11} a.f.s.).

Procedure—To minimize the adsorption of the drug onto the walls of the glassware, all tubes were cleaned with chromic acid cleaning solution, rinsed with water, and dried. The clean tubes were siliconized using a 1.0% solution of dimethyldichlorosilane in toluene and air dried.

Blood samples were collected into heparinized syringes. The blood was immediately centrifuged to separate the plasma; the fresh plasma was placed in the freezer and stored at −20° until assayed.

The plasma samples from monkeys were analyzed in the same manner as samples prepared for the calibration curve, except that the addition of chlormadinone acetate was omitted.

A calibration curve was prepared by the following procedure: 5 ml. of human plasma[10], shown to be free of chlormadinone acetate and caproate, was pipeted into each of five, 20-ml. screw-cap centrifuge tubes. To separate tubes was added 0.1, 0.2, 0.3, 0.4, or 0.5 ml. of the standard solution of 50 ng./ml. of chlormadinone acetate. Thus, individual tubes contained 5, 10, 15, 20, and 25 ng. of chlormadinone acetate. The solutions were incubated at 37° for 15 min. After incubation, 0.5 ml. (125 ng.) of the mass internal standard solution of chlormadinone caproate was added to each tube. Each tube was thoroughly shaken for several minutes, then 1 ml. of a saturated aqueous solution of sodium carbonate was added, and the contents were intimately mixed. To the homogeneous mixture in each tube was added 9.5 ml. of spectroquality hexane. Again the tubes were vigorously shaken for 5 min. and then centrifuged at 3000 r.p.m. for 5 min. or until the phases separated. Approximately 8.5 ml. of the hexane phase was transferred to another 15-ml. centrifuge tube. Five milliliters of 0.5 *N* sodium hydroxide was added to the tube containing the hexane phase. The mixture was first shaken for 5 min. and then centrifuged for 5 min. at 3000 r.p.m. Finally, the hexane phase was transferred to a 15-ml. tapered centrifuge tube, and the solvent evaporated at 40° under nitrogen on the evaporator. The sides of each tube were washed with 1 ml. of spectroquality benzene and again evaporated to dryness. The washing was repeated. To each tube was then added 40 μl. of spectroquality benzene, the tube was sonorated for 15 sec., and 5 μl. of the resulting solution was injected on the chromatographic column. Data of a typical calibration curve are given in Table I. From the table it is evident that the instrument response is linear throughout the concentration range employed.

Following chromatography, the baseline was drawn and peak heights of chlormadinone acetate and caproate were measured. The ratios obtained by dividing the chlormadinone acetate peak heights by chlormadinone caproate peak heights were plotted on

[10] Obtained from the Lilly Clinic.

Table II—Precision and Accuracy in Measurement of Chlormadinone Acetate Added to Human Plasma

Study Number[a]	Volume of Sample, ml.	Number of Knowns	Weight of Chlormadinone Acetate (ng./ml.) Added	Mean	*RSD*, %	*RE*, %
1	5	5	1.00	1.02	±4.4	+2.0
1	5	5	3.00	3.48	±22.1	+16.0
1	5	5	5.00	5.78	±6.9	+15.4
2	5	5	0.50	0.57	±11.5	+13.2
2	5	5	1.50	1.51	±5.2	+0.7
2	5	5	3.33	3.26	±1.9	−2.1

[a] Study 1 was done on the W-98 column, and Study 2 was done on the SE-30–Carbowax 20M.

the ordinate of cartesian graph paper, and the weights in nanograms of chlormadinone acetate per sample were plotted on the abscissa. By using the peak height ratio of a sample and the calibration curve (Table I), the total weight of chlormadinone acetate in the sample was determined by reading from the ordinate to the abscissa. The weight of chlormadinone acetate per milliliter was obtained by dividing the total weight by the sample volume. A calibration curve was prepared each day. To obtain the precision and accuracy of the assay method, plasma samples prepared to contain known amounts of chlormadinone acetate were randomized and assayed with the collected plasma samples (Table II).

Extraction Studies—Chlormadinone acetate, tritium labeled at C_1 and having an original specific activity of 5 μc./mg., was purified on a silica gel plate by preparative TLC. The solvent system used was 30 parts ether to 70 parts chloroform. The chlormadinone acetate (R_f value 0.43 after two developments) was visualized with the aid of 254-nm. wavelength UV light, outlined, and scraped from the plate. Chlormadinone acetate was extracted from the plate scrapings with a 1:9 mixture of methanol–chloroform. The radiolabeled drug was determined to be chemically pure by IR spectroscopy and radiologically pure by TLC. The chlormadinone acetate thus obtained had a specific activity of 1.79 μc./mg. (mean of five studies, three samples each study; range 1.70–1.93).

This labeled chlormadinone acetate was added to plasma samples which were then equilibrated at 37° for 15 min. The concentrations used were either 10 or 100 ng./5 ml. of plasma. The plasma was extracted as already described. Samples of the plasma, the aqueous wash phase, and the final hexane phase were analyzed for radioactivity. These results are summarized in Table III. The plasma was combusted by the Schöniger procedure. The combustion residue was dissolved in 10 ml. of a solution composed of three parts of ethanolamine and seven parts of 2-methoxyethanol[11]. To this solution was added 10 ml. of toluene scintillator counting solution (10 ml. toluene with 0.5% 2,5-diphenyloxazole). The aqueous phase was added to a solution of equal volumes of 2-methoxyethanol[11] and toluene containing 0.5% 2,5-diphenyloxazole, while the hexane phase was evaporated and the residue taken up in toluene containing 0.5% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene. The resulting solutions were counted on a liquid scintillation spectrometer[12]. Corrections for quenching were made by the internal standardization and channel ratio method.

Preparative GLC—The chromatograph was equipped with a 61.0-cm. (2-ft.), 3.8% W-98 on Diatoport S column, a flame-ionization detector, and a gas stream splitter system. The carrier gas flow (helium) was approximately 100 ml./min., while the temperature was 215° as used for the electron-capture system. Chlormadinone acetate was injected onto the column, and the material eluted was collected in a capillary tube at room temperature. The contents of the capillary tube were analyzed by mass spectroscopy and found to be identical with authentic chlormadinone acetate. Thus, chlormadinone acetate is stable under the GLC conditions employed in this study.

Drug Identification—Unfortunately, little is known with respect to the metabolic fate of chlormadinone acetate; therefore, none of the metabolites was available as reference materials.

[11] Methyl Cellosolve.
[12] Packard Tricarb model 3380.

Table III—Recovery Studies of Chlormadinone Acetate-C_1-^{3}H Added to Human Plasma

Study Number	Weight of Chlormadinone Acetate C_1-^{3}H (ng./ml.) Added	Percent Distribution of Radioactivity[a] Extracted Plasma	Sodium Hydroxide Wash	Lost in Dry Down	1–10 ml. Hexane	2–5 ml. Hexane
1	100	2	<0.5	4.5	54	—
2	100	—	—	—	56	—
3	100	—	—	—	—	69
4	100	—	—	—	—	74
5	10	—	<0.3	—	65	—
6	10	—	<1.0	—	—	77
7	10	—	<1.5	—	58	—
8	10	—	<1.0	—	—	72

[a] These results are not corrected for mechanical loss, emulsion loss, *etc.* The actual extraction efficiency for the drug into hexane from water was determined to be >95%.

The mass spectrum of the plasma extract of a monkey that had been dosed with 100 mg. of chlormadinone acetate displayed too much background interference to prove conclusively that the GLC peak measured during assay was chlormadinone acetate. Chlormadinone, the corresponding alcohol (6-chloro-4,6-pregnadien-17α-ol-3,20-dione), was also detected with equal ease by GLC with electron capture, and the retention time was slightly less, under the conditions specified, than that for the acetate ester. Mixtures of pure alcohol and acetate indicated that when the alcohol content was raised beyond 25%, a shoulder was displayed on the leading edge of the ester peak.

In further studies, another monkey was dosed by mouth with 8.18 μc. (approximately 2 mg.) of C_1-tritium-labeled chlormadinone acetate. A total of 10 ml. of plasma was collected 4 hr. after dosing. Two 5-ml. portions of plasma were extracted and washed as previously described, except that the addition of 0.5 ml. of the mass internal standard solution of chlormadinone caproate was omitted and 10 ml. of spectroquality hexane was used. After the evaporation and washdown steps were completed, the dried residue was dissolved in 250 μl. of chloroform with the aid of a sonorator and the entire solution was spotted on a preparative silica gel plate with fluorescent indicator along with 100-mcg. samples of chlormadinone (alcohol) and chlormadinone acetate in adjacent lanes. This plate was developed twice with a solvent of ether–chloroform (30:70).

The silica gel from areas 1.5 × 2 cm. adjacent to that area occupied by the unlabeled chlormadinone and chlormadinone acetate were scraped from the plate, and each was placed in a counting vial with 10 ml. of toluene containing 0.5% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene. The vials were well shaken, the silica gel was allowed to settle, and the supernatant liquid was counted on the liquid scintillation spectrometer. After corrections for quenching, these data indicated that about one-third of the tritium-labeled material was moving with the mobility of chlormadinone and two-thirds of the tritium-labeled material was moving with the mobility of chlormadinone acetate. This study indicates that some chlormadinone is probably being measured with and reported as chlormadinone acetate.

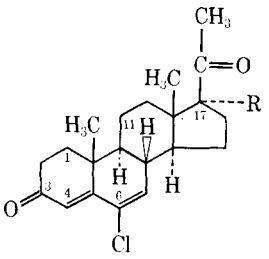
RESULTS AND DISCUSSION

From Table IV, it is evident that the peak plasma concentration of chlormadinone acetate occurs in monkeys from 3 to 6 hr. after

Table IV—Concentration of Chlormadinone Acetate Found in Plasma from Monkeys

Animal Weight, kg.	Tablet Dose, mg.	Sex	Time, hr. 0[a]	1	2	3	4	6
			Weight of Chlormadinone Acetate, ng./ml.					
3.1	2.0	M	0.0	6.2	12.5	16.8	12.8	—
2.8	2.0	F	0.0	7.2	11.5	10.8	13.2	—
4.4	1.0	M	0.0	—	3.7	—	6.9	4.2
3.8	0.5	F	0.0	—	4.5	—	5.8	1.4

[a] No GLC peak was ever observed in the region of chlormadinone acetate in the blank plasma samples obtained from monkey or human (5).



I: R = $OCOCH_3$
II: R = $OCOCH_2(CH_2)_3CH_3$

administration of the medication. In the monkey studies, the concentration of the drug found in the plasma was proportional to the dose administered. However, the 2-mg. dose did not give four times the plasma concentration obtained from the 0.5-mg. dose. In the published human data (5), the peak concentrations appeared 2–3 hr. postadministration.

Data in Table II indicate that the method is reliable and reproducible within ±20% of theory with both columns. However, it can be easily seen that the mixed phase column gave better overall accuracy and precision than did the W-98 phase. This result is readily explained on the basis of interference of contaminants present in the plasma. The data obtained using the mixed phase column show the method to be reproducible at a concentration of 0.5 ng. chlormadinone acetate/ml. of plasma when a 5-ml. plasma sample is used.

The radioisotopic studies of extraction efficiency (Table III) show that one 10-ml. extraction gave approximately 58% recovery, while two 5-ml. extractions gave approximately 71% recovery. The data also show that little if any drug is lost during washing and that only a small percent is lost during the concentration stage. These two processes probably account for less than 5% loss. The majority of the drug lost can be attributed to the incomplete separation of the hexane phase from the aqueous phase due to emulsification at the solvent interface. Since special care must be taken so that none of the emulsion layer is transferred, no more than 8.5 ml. of hexane could be recovered of the 10 ml. added. Taking the mechanical losses into account, the extraction efficiency is greater than 95%. It also appears from Table III that a 10-fold variation in plasma concentration of drug has little effect on percent recovery.

Although the majority of steroids are very insensitive to electron-capture detectors, a few (*e.g.*, those containing a vinyl ketone function) do possess a reasonable amount of electron affinity. It has been shown that the vinyl ketone function has greater electron-capturing capability provided it has an opportunity within the molecule to interact further electronically (*e.g.*, to be further conjugated) (7). Thus, chlormadinone acetate, which has an additional double bond in conjugation with the vinyl ketone, is enhanced in electron-capture ability. The electron-capturing properties of chlormadinone acetate are even further enhanced by the presence of the chlorine atom at C_6. The formation of halogenated derivatives of steroids for the purpose of enhancing electron-capture ability is well documented in the literature (8). Thus, chlormadinone acetate is a structurally ideal steroid for electron-capturing ability.

The use of a 30.5-cm. (1-ft.) column was based on the following reason. At 220°, a silicone column gave a reasonable amount of bleed, which directly diminished the sensitivity of the electron-capture detector. However, after several days of conditioning, this bleed became minimal and the electron-capture detector gave reasonable sensitivity. Columns longer than 30.5 cm. (1 ft.) required higher temperatures; correspondingly, a higher bleed was observed and this greatly reduced the sensitivity of the electron-capture cell.

Recoveries of chlormadinone acetate from plasma were studied under a wide variety of conditions. Extraction from acidified plasma gave chromatographs in which excess background was observed; hence, poor results were obtained. On the other hand, extraction from plasma made basic with sodium hydroxide gave chromatograms with very little background. However, sodium hydroxide was unsatisfactory since it caused hydrolysis of the acetate moiety present in the drug. It was found, however, that a sodium hydroxide wash of the hexane extract was possible since hydrolysis of the drug occurred only in the aqueous phase. Extraction from plasma made basic with sodium carbonate resulted in chromatograms with interfering background intermediate between that obtained with plasma made basic with hydroxide and that obtained from acidified plasma. Thus, the carbonate-treated plasma extraction followed by a sodium hydroxide wash of the hexane phase was the method of choice.

Benzene, ethyl acetate, ether, chloroform, and methylene chloride could not be used as extraction solvents, since they extracted considerably more interfering material than did hexane. The halogenated solvents, of course, have electron-capturing ability and could conceivably interfere with the detection method if any residue remained.

Chlormadinone caproate was chosen as the mass internal standard, since it is very similar in structure to chlormadinone acetate and hence would be expected to be very similar in solubility, extractability, and electron-capture detection. The chromatographic retention time of the internal standard was also very favorable. Since the mass internal standard is added at the initial stage of the procedure, it compensates for variability in extraction and removes the necessity for quantitative transfers.

Analyses of plasma samples were performed on both the W-98 column and the mixed phase column. Although both columns gave satisfactory results, the mixed phase column was the one of choice. This column gave a greater sensitivity of detection primarily due to the fact that the peak corresponding to chlormadinone acetate was further removed from the descending portion of the background peak. The phenomenon of background influence on sample peak height observed during electron-capture analysis was adequately described by Rapp and Eik-Nes (9) and will not be discussed here.

Although radioisotope studies showed that two 5-ml. extractions of plasma with hexane gave better recovery than one 10-ml. extract, upon chromatography the two 5-ml. extractions also gave a larger amount of background than did the one 10-ml. extraction. In practice, it was observed that the two chromatograms appeared very comparable in chlormadinone acetate peak heights. Thus, on the basis of simplicity, the one 10-ml. extraction procedure was adopted.

If the pure drug in hexane was injected into the gas chromatograph, the limit of detection was found to be 0.20 ng. However, in actual plasma samples, the limit of detection was somewhat lower (~0.5 ng.) due to the background encountered which decreased the sensitivity of the cell.

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▲ To whom inquiries should be directed.