Table I—Analysis of Prepared Samples of Compound I by NMR

Sample	α-Form, mg	β-Form, mg	Percent α	Percent α Found	Percent Recovered
1	57.50	3.20	94.73	94.88	100.17
2	54.12	6.31	89.56	89.78	100.25
3	42.66	19.00	69.19	69.80	100.88
4	30.38	31.20	49.33	49.79	100.93
5	28.62	33.52	46.06	46.08	100.04
6	23.80	36.38	39.55	39.86	100.78
		Mean	100.51		
		SD	0.40		

protons is probably due to the difference in the geometry of the two diastereoisomeric pairs and the manner in which the lanthanide shift reagent contacts them. This produces a greater effect in one case than in the other.

Positive identification of each methyl doublet was determined (Fig. 4). Under the same conditions, the NMR signal for the protons of the methyl doublet of the β-form was shifted further downfield than was that for the α-form. This finding confirms what was apparent from the examination of Fig. 3 where the downfield doublet is much smaller than the one upfield.

Table I reports the results of NMR analyses of prepared samples of I. Figures 5 and 6 show the NMR spectra of one of these mixtures, their expansion, and their integration. The recovery studies of these samples resulted in a mean of 100.51% with a standard deviation of ±0.40. There appears to be a constant positive bias which could result from the slight contamination of the β-form with the α-form. This is quite reasonable because α was easily obtained in pure form while β was separated from α only with great difficulty.

Two commercial size batches of I were made by the same process and gave ratios of 83.3% α to 16.7% of β and 82.9% α to 17.2% of β, respectively. The overall purity of these batches was 99.7% as determined by the method given in the *Experimental* section.

The accuracy and simplicity of this NMR method make it very useful and rapid for determining the relative proportions of the diastereoisomeric pairs of I.

REFERENCES

(1) D. B. Reisner and B. J. Ludwig, *J. Heterocycl. Chem.*, **6**, 953(1969).

(2) D. B. Reisner, B. J. Ludwig, H. M. Bates, and F. M. Berger, U.S. pat. 3,598,814 (1971).

(3) C. van der Vlies, G. A. Bakker, and R. F. Rekker, *Pharm. Weekbl.*, **101**, 93(1966).

(4) C. C. Hinckley, *J. Amer. Chem. Soc.*, **91**, 5160(1969).

(5) J. K. Sanders and D. H. Williams, *Chem. Commun.*, **1970**, 422.

(6) R. E. Rondeau and R. E. Sievers, *J. Amer. Chem. Soc.*, **93**, 1522(1971).

ACKNOWLEDGMENTS AND ADDRESSES

Received February 21, 1974, from the *Analytical Research and Development Department, Carter-Wallace, Inc., Cranbury, NJ 08512*

Accepted for publication May 7, 1974.

Presented to the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, San Diego meeting, November 1973.

The authors thank Dr. D. B. Reisner for providing samples of I and its α- and β-diastereoisomeric pairs and for helpful discussions during the preparation of this manuscript.

[x] To whom inquiries should be directed.

Progesterone Injection Assay by Liquid Chromatography

ROBERT H. KING, LEE T. GRADY [x], and JEANNE T. REAMER

Abstract □ An assay was developed for the progesterone content of injections composed of refined, fixed oils as the primary solvent. Reversed-phase, high-pressure liquid chromatography separates the progesterone from all other known ingredients following initial extraction into aqueous ethanol. Methyltestosterone was found to be a desirable internal standard, and peaks were measured photometrically. Twelve injection samples and six standards can be completed in 1 day. A system suitability test was defined. Evidence is presented for the recovery, specificity, accuracy, and precision of the method.

Keyphrases □ Progesterone injection—analysis by high-pressure liquid chromatography □ Steroids—analysis of progesterone injection by high-pressure liquid chromatography □ High-pressure liquid chromatography—analysis, progesterone injection

The official assay for progesterone in oil is a gravimetric determination of the double 2,4-dinitrophenylhydrazone adduct (1). This assay is unspecific, measuring any ketosteroid content, and serious problems have been observed with the quality of the precipitating reagent and with formation of the precipitate. A new assay with notably improved specificity was required in light of apparent problems with marketed items.

Analytical approaches to drugs in oil-based pharmaceuticals (2) and the pharmaceutical aspects of high-pressure liquid chromatography (HPLC) (3) have been reviewed. Specific, but cumbersome, progesterone determinations have been advanced including an IR partition column assay (4) and an extraction method followed by GLC (5). The purposes of this study were to develop and to define a specific and simple assay that utilizes the advantages offered by HPLC in the analysis of steroids.

EXPERIMENTAL

All reagents were USP, NF, or ACS grade. Spectroquality 2-propanol was purchased. Samples of commercial progesterone injection NF were obtained locally and from manufacturers. Fixed oils were obtained locally.

Chromatographic System—The instrument[1] used a 1-m by

[1] DuPont model 830 high-pressure liquid chromatograph, fitted with circulating warm-air oven and low-pressure mercury UV photometer.

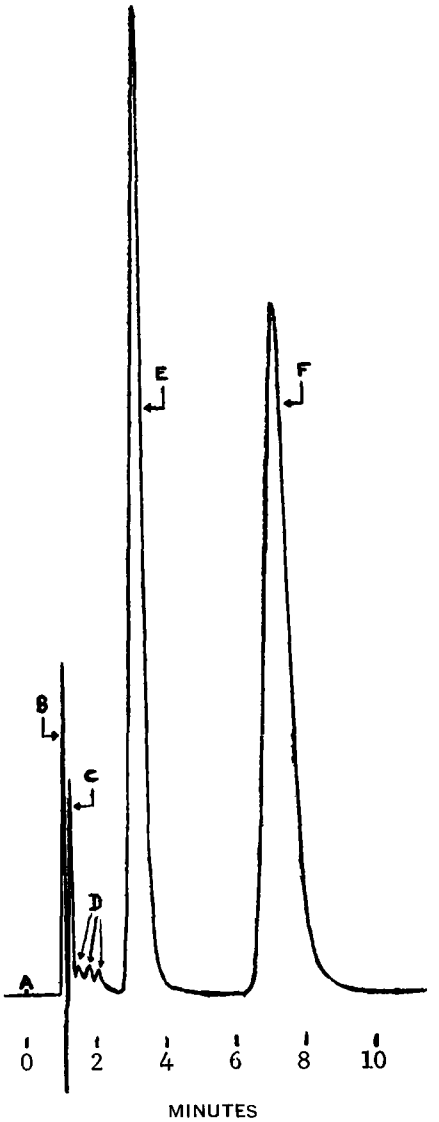

Figure 1—*Product A, 50 mg/ml through procedure. Key: A, inject; B, extracted material from injectable; C, solvent; D, minor impurities; E, methyltestosterone; and F, progesterone.*

2.1-mm i.d., stainless steel column packed with spherical glass microbeads, nominally 30 μm in diameter, with a thin porous coating to which is chemically bonded 1% (w/w) octadecylsilane[2]. The mobile phase was degassed, membrane-filtered[3], 18% 2-propanol–distilled water (v/v) at a flow rate between 0.8 and 1.2 ml/min at about 42–44°. A low-pressure mercury, 254-nm photometer was used as the detector, and an electronic digital integrator[4] was used for area and retention time data.

Internal Standard Solution—Transfer about 165 mg of NF methyltestosterone reference standard, accurately weighed, into a 25-ml volumetric flask, add sufficient 85% ethanol to dissolve it, dilute to volume with 85% ethanol, and mix.

Assay Preparation—Transfer, by calibrated tuberculin syringe, a volume of progesterone injection equivalent to about 25 mg of progesterone into a 15-ml Teflon-lined screw-capped centrifuge tube. Extract with three 5-ml portions of 85% ethanol, shake gently for 10 min, centrifuge[5] for 15 min or until the alcohol layer is clear, and carefully transfer the upper alcoholic layers by suitable Pasteur pipet to a 25-ml Teflon-lined screw-capped test tube, taking care to rinse the transfer device with about 1 ml of 85% ethanol after the first extraction. To the combined alcohol layers, add 2.0 ml, by pipet, of internal standard solution and mix well to obtain about 18 ml of a solution approximately 1.39 μg/μl in progesterone and 0.62 μg/μl in methyltestosterone.

Standard Curve—Transfer four portions of NF progesterone reference standard, about 20, 22.5, 27.5, and 30 mg, accurately weighed, to individual Teflon-lined screw-capped centrifuge tubes and dissolve each in about 15–16 ml of 85% ethanol. Add 2.0 ml of the internal standard solution by pipet and mix. Inject 5 μl from each solution into the chromatographic system. Determine the peak area or heights of the progesterone, P_p, and methyltestosterone, P_{mt}, and calculate the ratio, R_s, by the formula P_p/P_{mt}. Plot the standard curve of the R_s values against the amount, in milligrams, of NF progesterone contained in each portion.

System Suitability—Chromatograph six to 10 injections of a standard solution, containing approximately 1.5 mg/ml progesterone and 0.75 mg/ml methyltestosterone, and measure the peaks as directed for the standard curve. The analytical system is suitable for conducting the assay if: (*a*) the relative standard deviation (*RSD*), calculated by the formula 100 × (standard deviation/mean ratio), for the ratio R_s does not exceed 1.5%; (*b*) the resolution factor between peaks is not less than 3.5; (*c*) the tailing factor (sum of the distances from peak center to the leading edge and to the tailing edge divided by twice the distance from peak center to the leading edge), measured at 5% of peak height of the progesterone

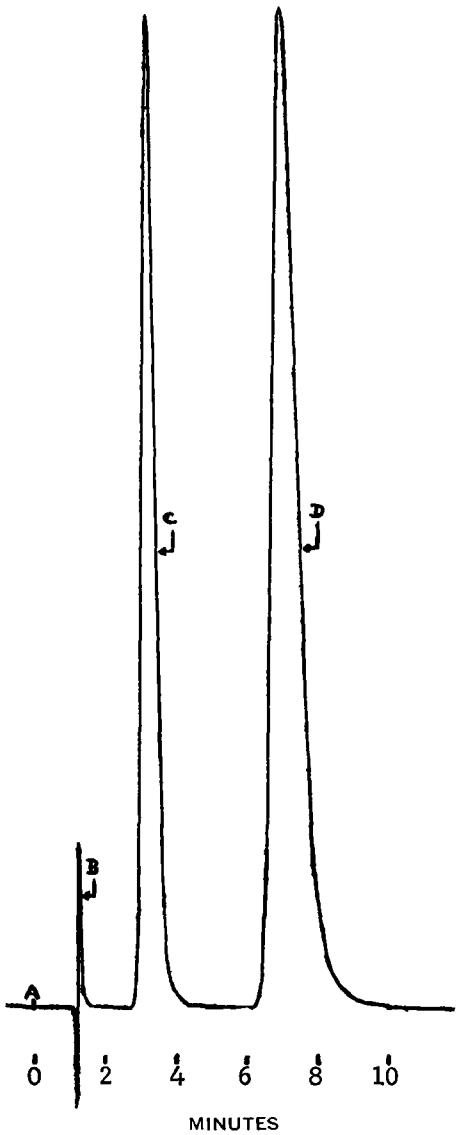

Figure 2—*Standard solution. Key: A, inject; B, solvent; C, methyltestosterone; and D, progesterone.*

[2] ODS Permaphase on Zipax, from the Instrument Products Division, DuPont and Co., Wilmington, Del.
[3] Gelman DM Metricel, 0.45 μm.
[4] Infotronics CRS-204 digital integrator.
[5] International model UV was used at about 1000 RCF.

Table I—Assay Results[a]

Manufacturer, Vial Volume, ml	Labeled Strength, mg/ml	HPLC[b] Peak Areas Mean Recovery, %	*RSD*, %	*n*	HPLC[b] Peak Heights Mean Recovery, %	*RSD*, %	*n*	Gravimetric[c,d,e] Mean Recovery, %	*RSD*, %	*n*
A[f] 1	25	78.4	0.23	4	79.5	0.48	4	91.2	0.95	4
								85.9[h]	0.80	6
A[g] 1	50	84.0	3.10	3	84.9	0.24	4	83.1	0.80	2
		86.4	1.48	3	85.4	0.65	3	—	—	—
B 10	25	90.9	0.60	4	90.4	0.17	4	86.4	0.46	3
		90.2	0.06	3	90.1	0.06	3	—	—	—
B 10	50	100.4	0.81	4	100.8	0.36	6	100.9	1.87	3
		100.7	0.29	4	100.4	0.46	5	—	—	—
C 10	25	101.7	0.33	7	102.1	0.56	7	100.5	0.45	3
		101.5	0.60	5	101.6	0.34	5	—	—	—

[a] As percent of label claim. [b] *n* is the number of repetitive injections of assay preparation. [c] Reference standard in sesame oil, 25.03 mg/ml, yielded a mean recovery of 100.3% with an *RSD* of 0.56% (six determinations), independent of lot of reagent. [d] Matheson, Coleman and Bell reagent, 1972. [e] *n* is the number of times the injection was assayed. [f] Small amount of extraneous material was observed on solvent front tail, but it did not interfere with quantitation. [g] Area ratios (by integrator) affected by extraneous material present on solvent front tail. [h] Eastman reagent, 1968 and 1970.

Table II—Standard Recovery from Oil[a,b,c]

Progesterone, mg	Sample	Peak Areas Recovery, %	Mean, %	*RSD*, %	Peak Heights[d] Recovery, %	Mean, %	*RSD*, %
15	1	98.9, 99.3	99.9	0.97	100.5, 100.9	100.8	0.30
	2	100.6, 100.9			101.2, 100.7		
25	1	98.9, 99.1, 99.6	99.2	0.36	99.8, 99.6	99.7	0.42
	2	98.7, 99.2, 99.0			100.2, 100.1, 100.0		
	3	98.8, 99.1			98.9, 99.0		
	4	99.4, 99.2, 99.2			99.5, 99.7, 99.5		
35	1	99.4, 99.8	100.0	0.56	100.1, 100.4	100.2	0.21
	2	100.2, 100.7			100.4, 100.0		
40	1	98.9, 99.5	99.7	0.60	99.1, 99.6	99.7	0.60
	2	99.9, 100.3			99.5, 100.5		
50	1	100.3, 99.9	99.9	0.37	99.7, 100.1	100.4	0.65
	2	99.4			100.6		
	3	100.0			101.2		
	2[e]	99.1, 99.3	99.4	0.30	99.8, 99.7	100.0	0.47
	3[e]	99.3, 99.8			100.7, 99.8		
60	1	100.2, 100.9	100.6	0.49	100.8, 100.7, 101.9	101.1	0.66
Total[f]			99.9	0.45	—	100.3	0.57

[a] Progesterone weighed directly into centrifuge tube and dissolved in sesame oil. Samples extracted and compared to nonextracted standards (injected in duplicate), using slope of resultant graph to convert ratios to milligrams progesterone; samples prepared over a 2-day period. [b] Except where noted, 2.0 ml of internal standard solution was added. [c] Area ratios of same solutions did not change over ~6-day period, but height ratios of the same solutions decreased ~2.5% over the 6-day period. [d] Minimum peak height (for 15-mg standards) approximately 8.4 cm. [e] 4.0 ml of internal standard solution added to these solutions to approximate 25-mg ratios. [f] Grand totals for recovery based on 2.0-ml internal standard addition only.

does not exceed 2.0; and (*d*) the capacity factor, *k'*, for the methyltestosterone is not less than 1.5.

Procedure—Inject 5.0 μl of the assay preparation into the chromatographic system and obtain a chromatogram and ratio as directed under for the standard curve. Read from the standard curve the quantity, in milligrams, of $C_{21}H_{30}O_2$ in the volume of the injection taken. The column should be rinsed with 100% 2-propanol for 30 min after ~40–60 injections to avoid any possible overloading of the column with extracted oil.

Tocopherols—To identify the extractable UV-absorbing oil constituents, an extract was prepared from equal volumes of oil and 85% ethanol. Thin-layer chromatograms of 500-μl streaks (under nitrogen) on fluorescent silica gel[6] were developed in (S_1) methanol–benzene (1:99) or (S_2) cyclohexane–ether (4:1). The bands were visualized with short wavelength (254 nm) UV light to simulate the detector of the high-pressure liquid chromatograph. The plates were then sprayed with antimony trichloride (6), antimony pentachloride (7), or phosphomolybdic acid (6) to observe color reactions. Individual bands visible under short wavelength UV light were scraped off the plates, extracted into 85% ethanol, filtered, and rechromatographed by HPLC to correlate the relative mobilities of the TLC bands with HPLC retention time as well as the color reaction to the spray reagents.

Only one band was observed to have a retention time similar to either methyltestosterone or progesterone. The relative TLC mobilities and reaction to the spray reagent suggested that this band and one other contained tocopherols. A better separation (8) of the tocopherols was achieved using (S_3) light petroleum–isopropyl ether–acetone–ether–acetic acid (85:12:4:1:1). Using this system, the two bands were separated into four components, all of which reacted similarly to the spray reagents as reported in the literature, and approximated the reported mobilities of α-, β-, γ-, and δ-tocopherols. The UV spectra of the isolated components were recorded and these were identical, having a minimum at 254 nm and maxima at 327, 287, and 236 nm, resembling the spectra of the tocopherols. δ-Tocopherol appears to constitute approximately one-third of the mixture. The solutions of each tocopherol were concentrated and analyzed by HPLC, and δ-tocopherol was confirmed to be the component of the oil that was being extracted and detected by HPLC just after the progesterone peak.

Progesterone Derivative—The 2,4-dinitrophenylhydrazone adducts of the progesterone in each commercial item and of the NF reference standard were examined by TLC using several systems, particularly chloroform–benzene (3:1) on silica gel. In each

[6] Merck silica gel F-254 precoated glass plates (Brinkmann), 250-μm layer.

Table III—Chromatographic Parameters (12, 14)

Column[a]	Peak	Retention Time, min	Capacity[b] Factor, k'	α[c]	Theoretical Plates, n[d]	Tailing Factor[e]	Resolution Factor, R_s[f]
I	Methyltestosterone	3.4	1.9	2.8	434	1.2	4.0
	Progesterone	7.4	5.3		470	1.7	
II	Methyltestosterone	9.8	4.5	3.7	204	3.1	3.6
	Progesterone	31.1	16.4		189	5.5	
III	Methyltestosterone	3.5	1.7	3.1	670	1.1	5.2
	Progesterone	8.2	5.3		661	1.3	
IV	Methyltestosterone	2.7	1.1	4.0	597	1.2	4.4
	Progesterone	6.8	4.3		355	1.6	

[a] I = ODS Permaphase, 44°, 18% 2-propanol–water, 75 atm; II = ODS Permaphase, 24°, 18% 2-propanol–water, 100 atm; III = second ODS Permaphase, 44°, 18% 2-propanol–water, 65 atm; and IV = HCP Permaphase, 34°, 17% 2-propanol–water, 110 atm. All were 1 m with DuPont Zipax as solid support. DuPont models 820 and 830 HPLC and UV 254-nm detector were used. [b] $k' = (T_r - T_0)/T_0$. [c] $\alpha = k'_2/k'_1$. [d] $n = 5.54\ [(T_r/(W_{h/2})]^2$. [e] Tailing factor = width at 5% peak height/2a, where a is the distance from peak center to the leading edge, measured at 5% peak height. [f] $R_s = [2(T_{r2} - T_{r1})]/[1.699(W_{1h/2} + W_{2h/2})]$, with retention and width in same graphical units.

case, only two spots, the known (9) *syn-* and *anti-*isomers, were observed and this finding was confirmed by UV and IR spectroscopy.

RESULTS AND DISCUSSION

Table I lists the results of assays of commercial items from three manufacturers. Substantial manufacturing problems apparently exist for this item (Table I and Fig. 1), both in potency and purity of ingredients. Both methods give accurate and precise assays of oil solutions of NF progesterone reference standard, but the more specific HPLC assay results are not always in agreement with the unspecific gravimetric assay results, particularly when subpotent samples are analyzed.

Quantitative Aspects—Replicate injections of a single solution of progesterone and methyltestosterone reference standards, 25 and 13.2 mg, respectively, in 17 ml of 85% ethanol, and calculation of R_s gave 0.34% *RSD* (n = 7) for electronically measured peak areas and 0.2% *RSD* for peak heights (Fig. 2). Replicate assays (n = 6) of a standard solution of 25 mg/ml progesterone in sesame oil[7] (7) also showed excellent precision, 0.43% *RSD* by area and 0.37% *RSD* by peak heights, and a mean recovery of 100.2% assayed against standard solutions in 85% ethanol. The stability of the system was indicated by the 0.5% *RSD* of a single standard solution over 1 day. It is concluded that the method is precise. Electronic area measurement is preferred for its speed and accuracy.

Recovery of an extracted standard compared to an unextracted standard was complete and unbiased (Table II) for the entire 15–60-mg/ml concentration range. Both the extracted and direct standard graphs showed a highly linear response, with linear regression coefficients ≥ 0.998 for each of nine completely separate standard graphs and no difference in either slope or intercept attributable to the extraction procedure. Chromatographic dynamics cause a slightly displaced y- (ratio) intercept (+5% for one octadecylsilane column and −4% for another, based on 25 mg ratio = 100%)—this would be an individual characteristic of such mixed absorption–partition columns so a standard graph rather than a one-point standard is required for maximum accuracy in the procedure. All progesterone is recovered (85.1, 13.1, 1.6, and 0.2% by HPLC assay of consecutive extractions), consistent with the fact that the slopes and intercepts obtained from extracted and nonextracted standards are identical.

Complete recovery of drug, in conjunction with the substantial specificity inherent in a chromatographic analysis, and the lack of significant known interferences define this as an accurate method.

Assay and standard preparations are stable in the screw-capped containers. Area ratios for 1-week-old solutions, both standard and sample, remained unchanged from the original ratio. Peak height ratios of the same aged solutions, however, showed an absolute 2.5% decrease in R_s at the end of 1 week, but the column at that point had an accumulation of uneluted oil on it. The same solution after 1 month on a freshly rinsed column (100% 2-propanol for 30 min) also gave the same peak height ratio as the original ratio (within 0.3%).

Hydroalcoholic extractants are well established (2, 5) in the recovery of polar drugs, including progesterone, from fixed oils. Hexane usually is mixed with the oil layer to effect a hexane–aqueous ethanol partition system for the oil *versus* the drug. The addition of a hydrocarbon was dispensed with for these reasons. After separation, the 85% ethanol layer is on top, the more convenient place for centrifuge tube methods; the oil readily breaks into fine droplets on shaking, and its viscosity is not so great as to present a mass-transfer problem during extraction. Small volumes were chosen to allow assay of unit doses, to avoid a concentration step in the procedure, and to benefit from centrifuge tube handling in preference to separators. Solubility of progesterone in the 85% ethanol extractant was not limiting, because a level in excess of 75 mg/ml at 24° was found, and the solubility[8] was still 33.5 mg/ml in the even more aqueous 70% ethanol. Additional partitions were necessary if the water content of the extractant was increased much beyond 15%.

Modification of the procedure can allow even faster analyses, such as single partitions directly into the internal standard solution with incorporation of oil solutions of the reference standard (or direct mixing). Conservation of reference standards can be effected by evaporating aliquots of progesterone extract and standard solutions and redissolving the residues in small volumes of the internal standard solution; *i.e.*, collect 3 × 5-ml extracts in a 25-ml volumetric flask and dilute to volume, transfer and evaporate a 2-ml (25-mg/ml) or 1-ml (50-mg/ml) aliquot, redissolve in 1 ml of 1.05 mg/ml methyltestosterone in ethanol, and inject 3 μl. Also, multiple weighings of progesterone can be replaced by a single solution of 2 mg/ml, pipeting 10–15-ml aliquots with dilution to be about 15–16 ml.

Chromatographic Aspects—HPLC is recognized as the method of choice for steroids. Resting on the extensive foundation forged by GC, applications of liquid chromatography to pharmaceutical problems have been precipitous (3, 10). Purity evaluation of progesterone has been suggested (11), using microparticulate silica adsorption chromatography, but the impurities (more polar) were in the tail of the progesterone peak which is less desirable in purity work. The reversed-phase column chosen for the assay reported here was selected precisely because of its known reliability and consistent success in steroid separations. Indeed, the retention and elution of progesterone in such a system already have been reported[9] (10).

The data in Table III were obtained with an octadecylsilane column, 18% 2-propanol–water, and ~1-ml/min flow. Chromatographic parameters are defined elsewhere (3, 12). Although ~35% ethanol–water or ~40% methanol–water on another octadecylsilane column was found satisfactory for chromatographic separation, 2-propanol–water is recommended due to the higher affinity of the oil for this alcohol, which makes column equilibration simpler after a 100% 2-propanol rinse. Another type of hydrocarbon polymer[10] (Column IV, Table III) also gave usable parameters, and a chromatographic precision of 0.6% *RSD* was obtained even at 22°

[7] Welch, Holme sesame oil, lot 5124A.

[8] N. Diding, O. Wallen, and B. Öhrner, personal communications.

[9] Unpublished Drug Standards Laboratory Report 71-735, February 8, 1972, "Progesterone, NF Reference Standard," used gradient elution of 2-propanol in water, 20–40%, and isocratic elution with 20% aqueous 2-propanol on ODS Permaphase, 55°.

[10] HCP Permaphase on Zipax, 1.0 m.

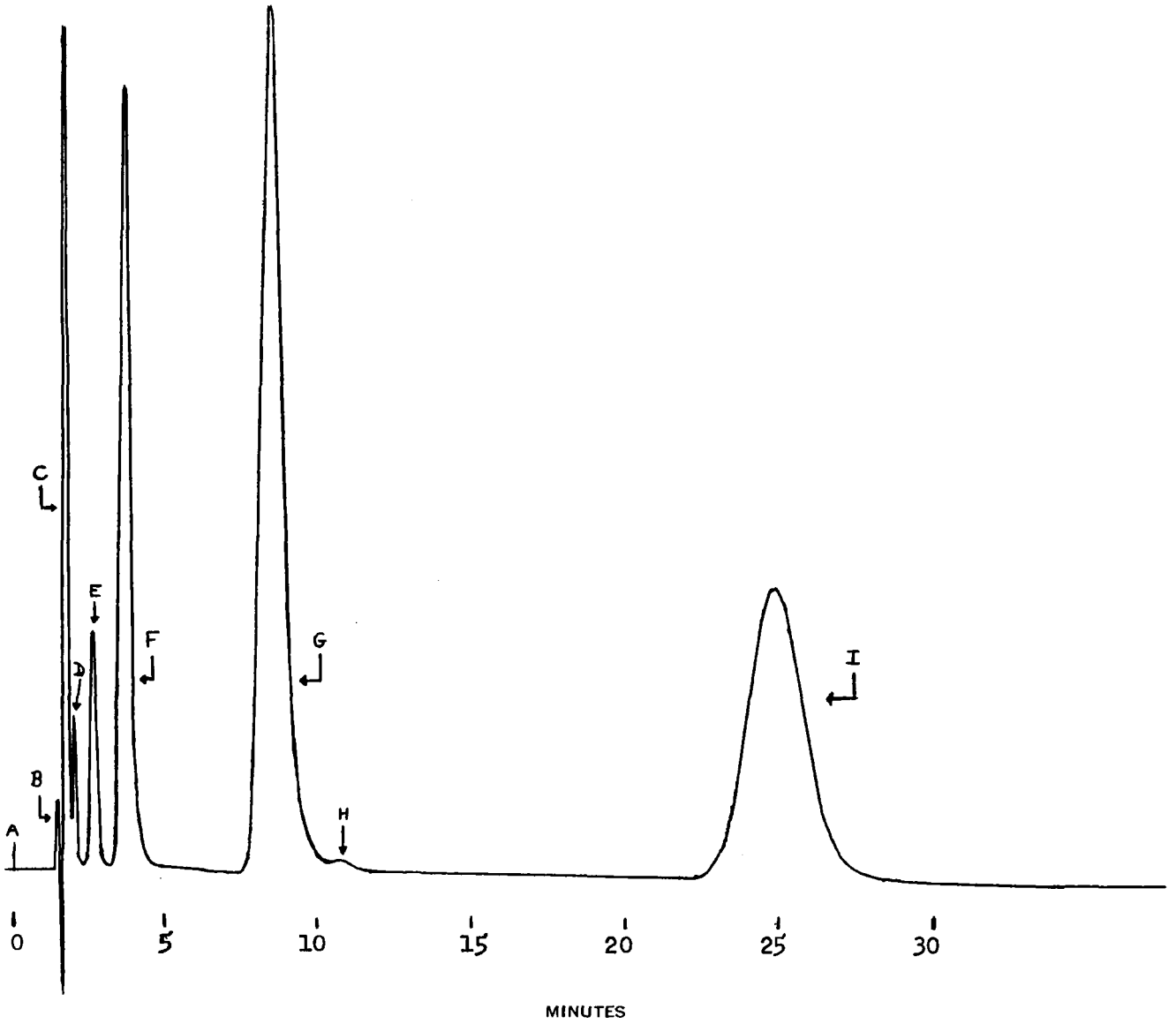

Figure 3—*Some components in oil injections. Extracted sample of "cold-pressed" oil which contained known components of commercial oil injectables. Key: A, inject; B, extracted material from sesame oil; C, solvent, benzyl alcohol, and phenol; D, impurity from benzyl benzoate; E, propylparaben; F, methyltestosterone; G, progesterone; H, δ-tocopherol,* k′ = ~*7.3; and I, benzyl benzoate,* k′ = ~*18.*

with this column (20% 2-propanol–water). Thus, this reversed-phase liquid chromatography approach promises substantial reliability and broad applicability.

Methyltestosterone was chosen as the internal standard because the chromophore and other molecular aspects were similar to progesterone, and it exhibited complete separation from the solvent front, progesterone, and oil impurities. Testosterone, in contrast, eluted too soon after the solvent effects on the baseline to allow reliable quantitation. About 20 steroid reference standards, many with the same chromophore, were examined to identify a suitable internal standard. As a corollary of the inherent specificity of the HPLC system, only a few [testosterone, norethindrone (k' = 1.0), methyltestosterone, and norethindrone acetate (k' = 5.3)] were retained beyond the solvent front and still eluted within a workable time frame. Methyltestosterone was the most desirable of these, being well separated from polar ingredients, progesterone, and oil components.

Solubility of sesame oil in 85% ethanol was found gravimetrically to be on the order of 1 mg/g, which predicts accumulation of about 4 μg of uneluted oil on the column per injection. In addition to the peak height ratio change mentioned, the retention times of progesterone and methyltestosterone were increased by approximately 30 sec during the 150 injections over 1 week's time, but with no apparent change in precision or recovery. Nevertheless, it is desirable to wash the column with 100% 2-propanol for 30 min after 40–60 injections to avoid loading the column with extracted oil.

System Suitability—A system suitability test was developed out of the experiences gathered during method development. In the present instance, more than one stationary phase may be suitable, although reexamination of other known components would be necessary before using a different packing. Furthermore, the analyst should have substantial additional flexibility in choosing temperatures, pressures, and concentrations of 2-propanol in water and still be able to establish a proven suitable system and an acceptable turnaround time.

System suitability testing was envisioned here several years ago as a mechanism to reconcile the requirement of highly defined methodology for official assays to the laboratory realities of wide variations in chromatographic supplies, instrumentation, range of operating conditions, and working times. The applicability of such tests, of course, extends beyond chromatography to other complex assays.

The underlying concept is that the chromatograph (make, plumbing, detectors, electronics, and recorder), packing (chemical type, source, batch, manipulations, and pretreatment), samples

(solvent, concentration, and injection size), operating conditions (temperature, flow rate, composition of mobile phase, and turnaround time), and even the analyst's technique constitute a *single system* and, as such, is amenable to an overall test of *system function*. Such a test reduces to a limited series of numbers and a precise, official language. Once a laboratory achieves a suitable system, straightforward performance of an assay procedure may be anticipated, presuming that classical chemical operations not covered in the test (extractions, concentrations, *etc.*) are performed normally. *In practice*, such testing must be based on adequate experience acquired during method development and evaluation with emphasis on the physical chemistry of chromatography; this probably is inherent in the work habits of experienced chromatographers. One other practical aspect is the presumption that the originating laboratory does not set forth a test based on such unusual materials, methods, or fortuitous chromatography that few other laboratories are likely to establish suitable systems.

Other Components—(See Fig. 3 for a composite.) A distinct advantage of this reversed-phase approach is that polar impurities (or perhaps decomposition products) of progesterone will elute first so that their presence is recognized without prolonging the turnaround time of the analysis. Indeed, one of the 50-mg/ml products, Product A, showed at least three minor polar components (Fig. 1). The product itself was distinctly more yellow or amber colored than the other commercial items or the various fixed oil samples. Expiration dates were not observed on most items. The identity of these components in commercal progesterone is not known with certainty, but work[11] is in progress in other laboratories which may establish their identity.

Benzyl alcohol, benzyl benzoate, phenol, ethanol, chlorobutanol, and propylparaben are the other labeled ingredients of the available pharmaceuticals tested. Chlorobutanol moves with the solvent front and is otherwise undetected. Benzyl alcohol and phenol also elute immediately and are detected by the UV photometer (Fig. 3). Benzyl benzoate elutes after progesterone (α = 3–4) in the octadecylsilane system and has, therefore, the effect of prolonging the turnaround time when present because it partitions into the 85% alcohol extractant. Propylparaben elutes just prior to methyltestosterone but gives baseline separation.

Normal, that is to say "refined," fixed oils[12] do not give any interfering peaks in this procedure, and presumably the compendial items are selected from refined fixed oils. One commercial product gave evidence of unrefined oil components, and a sample of sesame oil[7] of undetermined status was found to yield a small peak just after the progesterone peak which, if unobserved, could bias the (uncorrected) assay of the 25-mg/ml item by +0.3% if measured by electronic area devices with certain settings. Peak height data would not be affected to the same extent, depending on the efficiency and α-values of individual columns. To test the worst possible situation, a sample of health-food (unrefined and, therefore, noncompendial) sesame oil[13], so-called "cold-pressed" oil, was obtained, and a potential error of about +0.95% on progesterone data for the low-strength item was observed (Fig. 3). TLC of the oil allowed collection of the individual bands. Correlations were made between TLC and HPLC mobilities, and color reaction and UV spectra were determined. The interfering component appears to be δ-tocopherol. More polar oil constituents, *i.e.*, those less mobile on silica gel than tocopherols, all eluted in the solvent front in this reversed-phase liquid chromatography system. Van Niekerk's (13) liquid chromatography–adsorption system would be preferred for tocopherol analysis by HPLC. Stowe's (8) thin-layer system was found to be effective for separating the four tocopherols.

[11] E. Smith, personal communication.

[12] Such as Wesson or Kraft food-grade oils from cottonseed and soybeans and Planters peanut oil.

[13] Hain cold-pressed sesame oil, purchased from Mother Nature's Store, McLean, Va.

REFERENCES

(1) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, p. 598; *USP XIX Comment Proof*, 1, 1330(1973).

(2) E. Smith, *J. Amer. Oil Chem. Soc.*, **49**, 409(1972).

(3) A. F. Michaelis, D. W. Cornish, and R. Vivilecchia, *J. Pharm. Sci.*, **62**, 1399(1973).

(4) J. Wolff, *ibid.*, **52**, 93(1963).

(5) G. Cavina, G. Moretti, and P. Siniscalchi, *J. Chromatogr.*, **47**, 186(1970).

(6) "Pharmaceutical Applications of Thin-Layer and Paper Chromatography," K. Macek, Ed., Wiley, New York, N.Y., 1971, pp. 481–484, 650, 668.

(7) "Thin-Layer Chromatography," E. Stahl, Ed., Springer-Verlag, New York, N.Y., 1964, pp. 283–288.

(8) H. Stowe, *Arch. Biochem. Biophys.*, **103**, 42(1963).

(9) D. J. Watson and E. B. Romanoff, *J. Chromatogr.*, **22**, 192(1966).

(10) F. Bailey and P. N. Brittain, *ibid.*, **83**, 431(1973).

(11) D. R. Baker, R. A. Henry, R. C. Williams, D. R. Hudson, and N. A. Parris, *ibid.*, **83**, 233(1973).

(12) J. J. Kirkland, "Modern Practice of Liquid Chromatography," Wiley, New York, N.Y., 1971, pp. 10–15.

(13) P. J. Van Niekerk, *Anal. Biochem.*, **52**, 533(1973).

(14) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, pp. 787–789.

ACKNOWLEDGMENTS AND ADDRESSES

Received March 19, 1974, from the *Drug Standards Laboratory, 2215 Constitution Avenue N.W., Washington, DC 20037*

Accepted for publication May 10, 1974.

The Drug Standards Laboratory is sponsored jointly by the American Medical Association, the American Pharmaceutical Association Foundation, and the United States Pharmacopeial Convention.

[x] To whom inquiries should be directed.