Table X—Effect of Treating Decomposition Data According to Different Mathematical Models, Namely Zero, First, and Second Order[a]

	Order		
	Zero	First	Second
A, hr^{-1}	$(7.0 \pm 7.0) \times 10^6$	$(1.3 \pm 0.1) \times 10^{13}$	$(1.6 \pm 0.5) \times 10^{19}$
Ea, kcal $mole^{-1}$	11.2 ± 0.7	20.09 ± 0.05	28.7 ± 0.2
Absorbance error	± 0.021	± 0.0029	± 0.010

[a] In this case, the data confirm literature reports stating the decomposition is first order.

case, the dependent variable (c_i) is measured using a UV spectrophotometer. The reported uncertainty in such an instrument is ±0.005 absorbance unit[5], which represents a constant absolute error. Therefore, it is more correct to use the absolute absorbance values than the log absorbance. In a least-squares procedure, the former approach would minimize as far as possible to a constant absolute error, whereas the latter minimizes toward a constant relative error. Log weighting of the absorbance readings invariably returned a slightly lower value for the activation energy than when the absolute values were used and often produced nonrandom residuals. The biggest factor against using log absorbance in this nonisothermal technique is the comparatively larger relative standard deviations for the two parameters (Table IX).

The nonisothermal approach is very sensitive to the order of the reaction, so prior isothermal experiments need not be made to establish order. Data can be submitted for analysis, and the consequences of treating a reaction by the wrong mathematical model are clearly pointed out (see Fig. 1 and Table X). In this instance, an examination of the residuals clearly shows the reaction is first order, and this is substantiated by the smaller parameter and absorbance error standard deviations.

[5] Manual, Perkin-Elmer model 124 double-beam spectrophotometer.

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NOTES

Quantitative GLC Analysis of Plasma Cholesterol

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Abstract □ A GLC method is described for the quantitative analysis of cholesterol in plasma. The method was applied to the analysis of total cholesterol in 152 human plasma samples and compared with the results obtained by a standard automated colorimetric procedure on the same samples with values ranging from 100 to 472 mg %. The results of the two methods when subjected to a linear regression analysis yielded a sample correlation coefficient of 0.969 and a standard error of the estimate of ±15.2. The precision of the GLC method was determined by repeated total cholesterol analysis of the plasma of three human subjects. Similar results were obtained with all three. One of these had a mean total cholesterol of 172 mg % in 19 determinations with a standard error of the mean of ±1.0. Results with a single sample may be obtained in less than 2 hr, and one technician may obtain the results on 40 samples in 1 day using manual techniques. The GLC procedure clearly separates cholesterol from desmosterol and lanosterol. Adjustments in the volume and type of solvents allow quantitative determination of as little as 1.0 μg/sample. Evidence indicates that the method may be used for determining nonesterified cholesterol in the plasma.

Keyphrases □ Cholesterol—quantitative GLC analysis in plasma, compared to colorimetric method □ GLC—analysis, cholesterol in plasma, compared to colorimetric method

The determination of cholesterol in plasma is most often accomplished by procedures that require strong acids and/or ferric chloride to yield colored products. These methods measure the total cholesterol con-

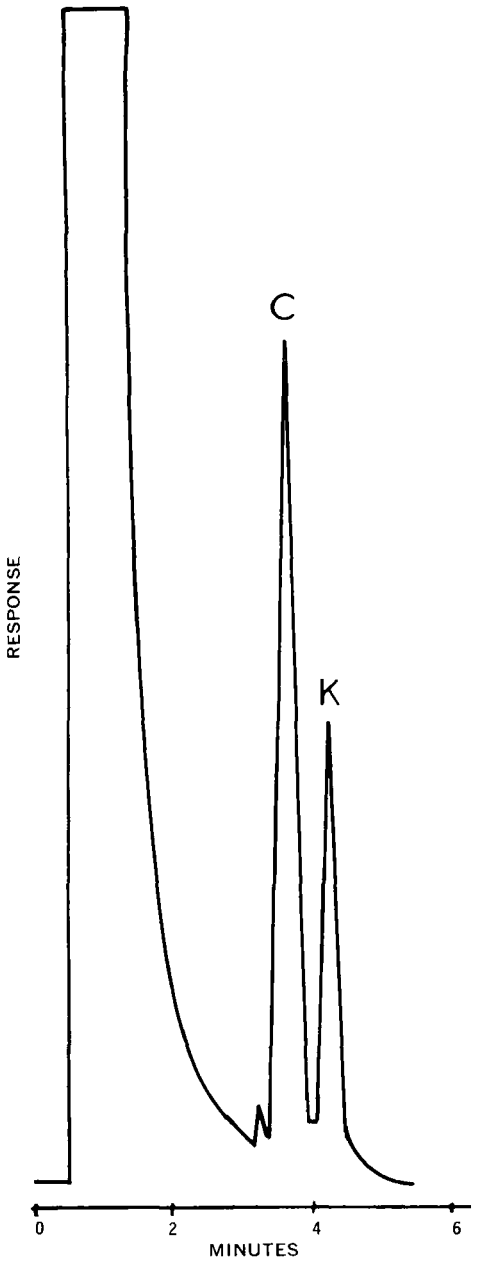

Figure 1—*Typical chromatogram resulting from an injection of an extracted plasma sample. Key: C, cholesterol; and K, internal standard, 11-ketoprogesterone. See text for details.*

tent, that is, both the nonesterified and esterified cholesterol, unless prior isolation of nonesterified cholesterol is carried out by precipitation with substances such as digitonin or tomatin. These methods, as well as others, have been discussed in detail (1). Most methods lack specificity, do not distinguish between esterified and nonesterified cholesterol, or do not have a high degree of sensitivity. For many purposes, of course, these limitations are not important, particularly when the simplicity and automation of some methods are considered. In this report, a GLC method for cholesterol analysis is described. Since the completion of this work, another publication has described a new improved GLC method for serum cholesterol (2). The present method contains some details and different conditions that may be advantageous or supplementary to this earlier report (2). The method has particular applicability in laboratories conducting cholesterol analysis occasionally, when a high degree of sensitivity is required, or when cholesterol is in the presence of significant quantities of very similar compounds such as desmosterol or lanosterol.

EXPERIMENTAL

Chemicals—All solvents used were reagent grade. The petroleum ether was the 30–60° bp fraction. Alcoholic potassium hydroxide was prepared by adding absolute alcohol to 60 ml of a 33% aqueous potassium hydroxide solution to make 1 liter. Cholesterol[1], 11-ketoprogesterone[2], cholesteryl acetate[3], lanosterol[3], and desmosterol[4] were used.

Instrumentation—Chromatography was performed with a gas chromatograph[5] equipped with a hydrogen flame-ionization detector. Prepurified dry nitrogen, 30 ml/min, was used as the carrier gas. A 1.8-m × 0.63-cm (6 ft × 0.25-in.) o.d. coiled glass column was packed with 3% OV-17 on Gas Chrom Q[6], 100–120 mesh. The column was initially conditioned at 375° for 30 min without carrier gas flow and then overnight at 325° with carrier gas flow and was subsequently operated at 300°. The temperature of both the detector and the injection port was 325°. When not in use, the column temperature was maintained at 200° to prolong the effective life of the column. Samples were injected with a 10-μl syringe[7].

Standard Solutions—A stock solution of cholesterol was prepared by dissolving 50 mg in 50 ml petroleum ether, bp 30–60°. Working standards were prepared by diluting the stock solution to concentrations of 2.5, 5, 10, and 20 mg/100 ml. An internal standard solution was prepared by dissolving 20 mg 11-ketoprogesterone in 50 ml acetone and adding petroleum ether to make 100 ml. Acetone was used because 11-ketoprogesterone is not sufficiently soluble in petroleum ether at this concentration.

Procedures—For the determination of total cholesterol, 1.0 ml of plasma was placed in a 70-ml culture tube with a Teflon-lined screw-cap, and the cholesteryl esters were saponified by adding 10 ml of alcoholic potassium hydroxide and incubating for 60 min at 45° in a slowly shaking water bath. After cooling, extraction was performed by adding 20 ml of petroleum ether. The tubes were shaken in a mechanical shaker for 5 min. To this solution, 10 ml of water was added and the mixture was shaken for 1–2 min. The mixture was then allowed to separate into two phases. Five milliliters of the petroleum ether fraction was transferred to a suitable glass tube and evaporated to dryness in a 75° water bath. The residue was dissolved in 2 ml of the internal standard solution, and approximately 1 μl of this solution was injected into the gas chromatograph. Peak height ratios of cholesterol–internal standard were used for quantitation.

Peak height ratios for known quantities of cholesterol were determined by pipeting 5 ml of each diluted standard solution into a suitable glass tube, evaporating this sample to dryness in a 75° water bath, and then dissolving the residue in 2 ml of the internal standard solution. These samples were injected in the same manner as the unknown samples. A standard curve was constructed by graphing peak height ratios of cholesterol–internal standard *versus* cholesterol concentration.

Variations of the procedure were developed for use with smaller plasma samples or biological samples containing smaller amounts of cholesterol. One such procedure consisted of removal of 15 ml of the petroleum ether extract. An appropriate quantity of internal standard was added and the entire volume was evaporated to dryness. The residue was dissolved in 100 μl of carbon disulfide and 1 μl was injected into the chromatograph. As little as 1 μg per sample may be quantitated with this procedure.

For the determination of nonesterified cholesterol, it is pro-

[1] Hormel Institute, Austin, Minn.
[2] Nutritional Biochemical Corp., Cleveland, Ohio.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Supplied by Dr. D. Dvornik, Ayerst Laboratories, St. Laurent, Quebec, Canada.
[5] Varian model 1800.
[6] Applied Science Laboratories, State College, Pa.
[7] Hamilton Co., Whittier, Calif.

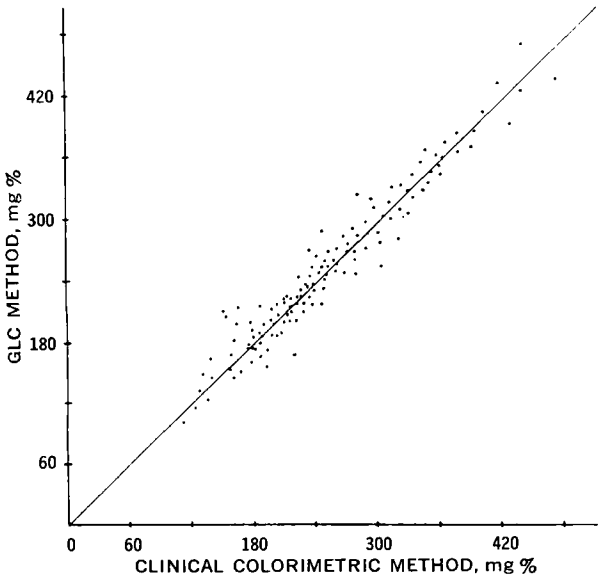

Figure 2—*Plot of the values of total plasma cholesterol performed by a clinical colorimetric method and the GLC method. The line represents a perfect correlation between the two methods. See text for details and statistical evaluation.*

posed that 95% alcohol be substituted for the alcoholic potassium hydroxide solution in the extraction and that the alcohol solution not be heated. Otherwise the procedure would be the same as described. To determine the stability of a cholesteryl ester with this procedure, cholesteryl acetate was dissolved in 95% alcohol in a concentration of 0.2 mg/ml, 10 ml of this solution and 1 ml of water were extracted with 20 ml of petroleum ether, and then 10 ml of water was added as before. Five milliliters of the petroleum ether layer was evaporated to dryness, and then 2 ml of acetone–petroleum ether (1:1) was added. One microliter was injected into the chromatograph to determine if any cholesterol was freed by hydrolysis of the cholesteryl acetate in the procedure.

Solutions of desmosterol and lanosterol were prepared in petroleum ether in concentrations of approximately 0.2 mg/ml. One microliter of these solutions was injected into the chromatograph to determine retention times relative to cholesterol.

Blood samples were obtained from 152 patients[8] for whom plasma cholesterol analysis had been ordered as part of a regular clinical workup. Venous blood samples were obtained and immediately heparinized. The sample was centrifuged, and the plasma was removed and subjected to analysis for cholesterol. Part of the plasma was sent to the clinical laboratory for total cholesterol analysis[9]. The rest of the plasma was subjected to the GLC method for total cholesterol analysis already described.

Three human subjects volunteered 40 ml of blood each from which the plasma was separated. Nineteen determinations of total cholesterol were performed on each volunteer's plasma, using 1 ml of plasma for each determination with the GLC method.

RESULTS AND DISCUSSION

A linear relationship was obtained when the peak height ratio of cholesterol-11-ketoprogesterone was plotted against cholesterol concentration over a cholesterol concentration range of 2.5–20 mg/100 ml in petroleum ether. These concentrations corresponded to total cholesterol plasma levels of 50–400 mg/100 ml with the extraction procedure described. The correlation coefficient of the peak height ratio to cholesterol concentration was 0.997, the standard error of the estimate was ±0.111, and the intercept was 0.034 with 16 determinations, four at each of four concentrations.

Similar linear standard curves were obtained with other ranges of cholesterol concentration in petroleum ether, including one range as low as 0.05–0.4 μg/ml. In this low range, 15 ml of standard petroleum ether solutions of cholesterol was evaporated along with an appropriate amount of internal standard solution and the residue was taken up in 100 μl of carbon disulfide by vortex agitation. Carbon disulfide was used as the solvent for injection into the chromatograph because of its low response in the flame-ionization detector. This allows detection of the very small quantities of cholesterol and internal standard injected (7.5–60 ng) at very sensitive electrometer settings. As little as 1 μl of plasma can be the sample size if this procedure is used. The sensitivity of this procedure compares quite well with those utilizing fluorometric techniques (3–5).

Table I—Reproducibility of Total Cholesterol by GLC

Plasma sample	1	2	3
Number of determinations	19	19	19
$\bar{X}$, mg %	161	172	184
SEM	±0.9	±1.0	±1.1
Range, mg %	153–170	162–181	176–192

A single peak with a retention time of 3.6 min was obtained for cholesterol in the gas chromatogram under the conditions stated. The internal standard, 11-ketoprogesterone, gave a single peak with a retention time of 4.2 min. A typical chromatogram from a plasma sample is shown in Fig. 1. Both peaks are quite symmetrical, and little interference in the plasma appears in the chromatogram. Progesterone has also been used successfully as an internal standard in the procedure with similar results to those obtained with 11-ketoprogesterone. It had a retention time of 3.1 min. Under certain circumstances it may be desirable to use progesterone instead of 11-ketoprogesterone for the internal standard.

Cholesteryl acetate had a retention time of 4.3 min under the GLC conditions described for cholesterol. No detectable cholesterol was liberated from the cholesteryl acetate during the extraction, evaporation, and injection procedures. Other endogenous cholesteryl esters should be as stable as the acetate and have even longer retention times. Therefore, the assay conditions described for nonesterified cholesterol appear to be reliable. Progesterone should be used as an internal standard rather than 11-ketoprogesterone since cholesteryl acetate has a similar retention time to 11-ketoprogesterone.

Desmosterol produced one major peak, with a retention time of 4.2 min, and two smaller peaks at 4.9 and 6.5 min. Obviously, progesterone is the internal standard to use rather than 11-ketoprogesterone in cholesterol assays where significant quantities of desmosterol may be encountered. Lanosterol injection gave two peaks of similar magnitude at 6.1 and 6.3 min. All peaks resulting from desmosterol and lanosterol injection are clearly separated and have longer retention times than cholesterol. Other GLC methods reported (2, 6–9) utilize SE-30 as the liquid phase. Utilization of this phase requires a much lower operating temperature, which leads to a lengthy retention time for cholesterol. A shorter column or a lower percent of liquid phase may be used to overcome this disadvantage, but resolving capacity is seriously lost. In this study, OV-17 was superior to SE-30 as a liquid phase for resolving cholesterol-related compounds. The multiple peaks seen after injection of desmosterol and lanosterol may have been due to impurities or thermal degradation.

The accuracy of the total cholesterol method reported here was assessed by comparing values with those obtained by a standard clinical laboratory method[9] on 152 human plasma samples. The values ranged from 100 to 472 mg % by the colorimetric procedure (Fig. 2). The line represents a would-be perfect correlation. The sample correlation coefficient obtained by a linear regression analysis of the two methods was 0.969, the standard error of the estimate was ±15.2, and the intercept was −7.14. The accuracy of the GLC total cholesterol method appears to be excellent, assuming that the colorimetric procedure measures a true value of total cholesterol.

To test the precision of the method, the GLC total cholesterol procedure was performed repetitively on each of three large plasma samples obtained from volunteers (Table I). The high degree of reproducibility indicates an adequate level of precision of the method for most purposes.

[8] Los Angeles County-University of Southern California Medical Center.

[9] By the Technicon N-24a procedure, an AutoAnalyzer procedure which utilizes a ferric chloride reagent.

The high temperatures used in the GLC method may lead to thermal degradation of cholesterol, especially since the 3β-hydroxyl group is not derivatized. However, no evidence for thermal degradation was observed in this study. A saturable adsorptive phenomenon for cholesterol in the column was not observed. Others reported this to be a problem (2, 6, 9). If loss on the column occurs in the method reported here, it does not manifest itself in poor accuracy or precision (Fig. 2 and Table I).

A technician may, starting with whole blood, obtain the results for total cholesterol on at least 40 samples in a working day. This assumes that manual techniques are utilized and only one column of a gas chromatograph is available. One column in these laboratories has been satisfactorily utilized for more than 1 year under the conditions specified.

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Correlation of Plasma Ibuprofen Levels with Biological Activity

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Abstract □ Investigations with ibuprofen [(±)-*p*-isobutylhydratropic acid], a well-tolerated, orally active, anti-inflammatory drug, were undertaken to: *(a)* determine the relationship among plasma drug concentrations, administered dose, and anti-inflammatory activity in developing and established polyarthritis in rats; and *(b)* compare the plasma drug disappearance half-lives in normal and polyarthritic rats. The results indicated that plasma drug concentrations in normal and polyarthritic rats were dose related. The logarithm of biological activity expressed as [% IPA/(100 − % IPA)], where % IPA is the mean percent inhibition of developing or established polyarthritis as measured by plasma inflammation units, was related to the logarithms of: *(a)* administered dose (mg kg^{-1}), *(b)* plasma drug concentrations (μg ml^{-1}) at 2 hr postadministration, and *(c)* average plasma drug concentrations (μg ml^{-1}) in a dosage interval at the equilibrium state. Half-lives for elimination of ibuprofen from the plasma of normal rats (after single-dose oral drug administration) and polyarthritic rats (after 29 doses) were essentially identical.

Keyphrases □ Ibuprofen—plasma levels correlated with biological activity □ Biological activity—correlated with ibuprofen plasma levels □ Anti-inflammatory agents—ibuprofen plasma levels correlated with biological activity

Ibuprofen[1] [(±)-*p*-isobutylhydratropic acid] (I) is a well-tolerated, orally active, anti-inflammatory agent utilized for the treatment of rheumatoid arthritis (1–3). The pharmacology, toxicology, and aspects of the absorption, distribution, and metabolism of I were reported previously (4–6). Recently, a sensitive and specific GLC procedure was described (7) for the determination of I in plasma. The present studies were conducted to: *(a)* determine the relationships among plasma I concentrations, orally administered dose, and anti-inflammatory activity in developing and established polyarthritis in rats; and *(b)* compare the elimination half-lives of I after single-dose administration to normal rats and after multiple-dose administration to rats with established polyarthritis.

EXPERIMENTAL

Rats with Developing Polyarthritis—Sixty Badger male rats (~235 g) were made polyarthritic by the intradermal injection into the tail of 0.5 mg of dead *Mycobacterium butyricum*[2] in 0.1 ml of mineral oil on Day 0. On Day 1, the animals were divided into six groups of 10 each. The groups received 0, 4.2, 9.0, 17.2, 33.6, or 67.2 mg I/kg, respectively, as a 1-ml aqueous suspension orally, bid for 14 days. On Day 15 all animals were weighed and scored. Five rats from each group received an additional (29th) oral dose of I, 2 hr prior to sacrifice. All animals were exsanguinated, and plasma specimens were obtained for the measurement of plasma inflammation units. Plasma aliquots from the animals receiving the 29th dose were stored at −18° until analyzed for I.

Rats with Established Polyarthritis—A series (180) of Badger male rats (~230 g) was made polyarthritic as described. On Day 14, the animals were visually scored, weighed, and divided into four groups of 45 each. Beginning on Day 15, the groups received 0, 16.4, 34.6, or 72.4 mg I/kg, respectively, as a 1-ml aqueous suspension orally, bid for 14 days. After 28 doses (Day 28), animals were visually scored and weighed. Beginning with the 29th dose (Day 29), two to five rats per group were sacrificed and exsanguinated at 0, 1, 2, 3, 4, 6, 8, 12, and 24 hr. Plasma specimens were

[1] Motrin, The Upjohn Co.; Brufen, The Boots Co. Ltd.

[2] Difco.