JOURNAL OF **Pharmaceutical Sciences**

November 1962 volume 51, number 11

Review Article

Gas Chromatography and Its Application to Pharmaceutical Analysis

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THE INTRODUCTION of chromatography as an analytical tool is generally attributed to Tswett (1) who coined the term chromatographic adsorption analysis. In principle, there appears to be no restriction on the nature of the mobile and the stationary phases in chromatography, nor on the types of equilibria involved. This was first recognized by Martin and co-workers (2, 3) who used a liquid adsorbed on a solid support as the stationary phase. The solid support might be a finely powdered inert material, such as diatomaceous earth, starch, or glass beads, or it might be sheets or strips of filter paper. In either case, separation took place by a partitioning of the solutes between two immiscible phases. In their first report on the partition chromatographic method, published in 1941, Martin and Synge (2) pointed out that the moving phase need not be a liquid, but might just as well be a gas. In fact, they even discussed the advantages that such a system might have. At the time nobody seemed to realize the tremendous significance of this suggestion, and the idea lay dormant for about 10 years until Martin, this time in cooperation with James, proceeded to work out the experimental technique. Their first paper on gas-liquid chromatography was published in 1952 (4). Actually, the principle of gas chromatography had been used for a long time for the separation of gases by means of a solid adsorbant (5, 6). However, it was the introduction of gas-liquid chromatography by James and Martin which led to the widespread acceptance of gas chromatography in general as an analytical technique. Gas-solid chromatography has remained the standard method for separation of permanent gases and low molecular weight hydrocarbons, while gas-liquid chromatography has become the method of choice for separation, identification, and quantitative determination of a wide variety of complex mixtures. The transition from gas-solid to gas-liquid chromatography is often gradual (7, 8) and, with the low concentrations of liquid phases now commonly used for separation of high boiling compounds, adsorption phenomena and the tailing which results are often difficult to eliminate.

The main advantages of gas chromatography over other modifications of chromatography, in fact over all other separation procedures, are speed, sensitivity, and versatility. The low viscosity of the mobile phase permits a high rate of flow and a fast equilibration between the thin film of liquid and the gas. As a consequence, separation can become very rapid. In many instances half a dozen or more substances may be separated in a matter of a few seconds. The sensitivity of the new ionization detectors is of the order of 10^{-12} Gm. per ml. of gas; 0.01 mcg. or even less can often be detected. A wide variety of stationary liquid phases, from nonpolar to very polar materials, are available for separation of a multitude of substances. Although essentially an analytical technique, gram quantities of very pure

Received from the School of Pharmacy, University of California Medical Center, San Francisco 22.
Supported in part by Grant M-3487 from the National Institutes of Health, Bethesda, Md.

materials can be isolated from high capacity columns.

LITERATURE

In spite of its recent origin, the literature on gas chromatography is already very extensive. More than 1600 papers were published in 1961 alone (9). In addition to the well-known abstracting periodicals dealing with chemical and related literature, two abstracting services devoted exclusively to gas chromatography are available (10, 11). Numerous review articles (9, 12–23), monographs (24, 25), and books (26–31) have been published. Papers presented at national and international symposia on gas chromatography have been collected and published in book form. The purpose of the present review is to present some of the fundamental aspects of gas chromatography in general and to deal in somewhat greater detail with its analytical applications with special emphasis on pharmaceutical analysis.

THEORETICAL PRINCIPLES

In gas-liquid chromatography, one is mainly concerned with the interactions between the solutes and the stationary liquid. There is only one liquid solution to be considered rather than two, as in liquid-liquid chromatography. This relative simplicity, coupled with the speed and reproducibility of gas chromatographic separations, has greatly stimulated an interest in the theoretical aspects of chromatography. Two approaches have been used to explain the various phenomena affecting retention, efficiency, and resolution. The "plate theory," first introduced by Martin and Synge (2) for liquid-liquid partition chromatography and developed further by Glueckauf (32), treats the chromatographic column as a discontinuous system analogous to a distillation column, involving a series of equilibria or theoretical plates. The "rate theory" (33–35) regards the chromatographic column as a continuous medium in which diffusion phenomena and mass transfer are taken into account. A thorough discussion of the theory of gas chromatography is beyond the scope of this review. The reader is referred to the original literature and to the several books and review articles on the subject.

Retention.—When vapors from a sample enter a gas chromatographic column they are retarded by the stationary phase and travel more slowly than the carrier gas itself. The rate at which each solute travels through the column depends, among other things, on its partition coefficient between the stationary liquid and the gas or, in other words, upon its vapor pressure above its solution in the liquid. The volume of gas passing through the column from the time of application of the sample to the emergence of the peak maximum is called the retention volume, V_R

$$V_R = t_R F_c \qquad \text{(Eq. 1)}$$

where t_R is the retention time measured from the time of sample injection to the appearance of the peak maximum, and F_c is the flow rate of the carrier gas measured at the outlet pressure and at the temperature of the column. The compressibility of the gas causes its linear velocity to increase along the length of the column due to a drop in pressure from the inlet to the outlet. The corrected retention volume, V_R^o, is calculated by introducing a correction factor, f, for the pressure drop in the column (4)

$$V_R^o = f V_R = f\, t_R F_c \qquad \text{(Eq. 2)}$$

where $f = {}^3/_2[(p_i/p_o)^2-1]/[(p_i/p_o)^3-1]$, p_i and p_o being the carrier gas pressure at the inlet and the outlet, respectively. Correction should also be made for the "dead volume" or "gas holdup" of the column, V_G^o. V_G^o may be estimated (36, 37) from the retention time of homologous compounds or measured as the corrected retention volume of a gas which will not be adsorbed by the column liquid, such as air

$$V_N = V_R^o - V_G^o = f\, t\, F_c \qquad \text{(Eq. 3)}$$

where V_N is the net retention volume, corrected for the pressure drop of the column, and t is the net retention time measured as the time between the air peak and the peak for the component being analyzed.

A fundamental equation relating the corrected retention volume to the partition coefficient and the amount of stationary liquid in the column is given by

$$V_R^o = V_G^o + K V_L = V_G^o + Kw/\rho \qquad \text{(Eq. 4)}$$

where K is the partition coefficient, V_L is the volume and ρ the density of the stationary liquid, both at the column temperature, and w is the weight of the liquid phase in the column. In order to obtain an expression for the retention volume that would be independent of the amount of liquid phase, Littlewood, Phillips, and Price (38) introduced the symbol, V_g, usually referred to as the specific retention volume. It is the net retention volume per gram of liquid phase reduced to standard temperature and pressure

$$V_g = 273\, V_N/Tw = 273\, K/T\rho \qquad \text{(Eq. 5)}$$

where T is the column temperature in °K.

Equation 4, relating the corrected retention volume to the product of the partition coefficient and the volume of stationary liquid, does not account for the changes in elution sequence which sometimes occur with changes in the concentration of liquid phase on the column. Martin (39) suggested that such changes, which are especially predominant with highly polar liquid phases, are due to adsorption of the solute on the surface of the liquid. He has modified Eq. 4 to include this phenomenon.

Column Efficiency.—The efficiency of a gas chromatographic column may, in the same way as a distillation column, be described in terms of its number of theoretical plates and calculated from the chromatogram by the expression

$$n = 16\,(t_R/b)^2 \qquad \text{(Eq. 6)}$$

where b is the base (in the same units as t_R) of the triangle formed by the tangents through the inflection points of the peak. The length of the column required to establish the equivalent of one equilibration between the gas phase and the stationary liquid is called "the height equivalent to a theoretical plate" (HETP). This value is obtained from the total number of plates (n) and the length of the column (l)

$$\text{HETP} = l/n \qquad \text{(Eq. 7)}$$

In linear, nonideal chromatography, such as gas-liquid chromatography, and in the absence of adsorptive effects, the peaks are symmetrical or nearly so, approximating a Gaussian distribution curve. However, the bands tend to broaden as they move down the column and the longer the retention time of a solute, the wider the emerging peak. This band broadening which reduces the efficiency of the column is due to several factors involving diffusion phenomena and resistance to mass transfer. Van Deemter, Zuiderweg, and Klinkenberg (34) have expressed the relationships as follows

$$\text{HETP} = A + B/u + Cu \qquad \text{(Eq. 8)}$$

or more fully

$$\text{HETP} = 2\lambda d_p + \frac{2\gamma D_{\text{gas}}}{u} + \frac{8}{\pi^2} \cdot \frac{K'}{(1+K')^2} \cdot \frac{d_f^2 u}{D_{\text{liq}}} \qquad \text{(Eq. 9)}$$

where λ is the statistical irregularity of the packing, d_p is the particle diameter of the support, D_{gas} and D_{liq} denote the molecular diffusion in the gas and the liquid phases, respectively, u is the average linear gas velocity of the carrier gas, γ is the labyrinth factor or tortuosity constant of the paths in the column, K' represents the product of the partition coefficient, K, and the ratio of the liquid volume to the gas volume of the column, and d_f is the mean diameter of the liquid film on the support.

The term A, referred to as "eddy diffusion," is due to the irregularities of stream paths in a porous medium. Van Deemter, *et al.* (34), found it to be independent of flow rate. It can be reduced by careful packing and by using uniform particle size of small diameter (40, 41). B represents molecular diffusion which is inversely proportional to the gas velocity, and C is concerned with resistance to mass transfer, expressing the lack of equilibration between the two phases. The effects of these factors are illustrated in Fig. 1 in which HETP is plotted against the average linear velocity of the carrier gas. The van Deemter equation has been widely discussed and there is general agreement that it presents a reasonably good picture of the conditions responsible for the spreading of chromatographic zones. However, recent work by several investigators has indicated a need for an additional term or terms similar to the C term, dealing with resistance to mass transfer in the gas phase (42, 43). The A term has also been the subject of much speculation and controversy (44, 45).

As expressed in the van Deemter equation and by several investigators, the apparent column efficiency is affected by the nature, flow rate, and pressure gradient (46, 47) of the carrier gas, the particle size of the support and the density of the

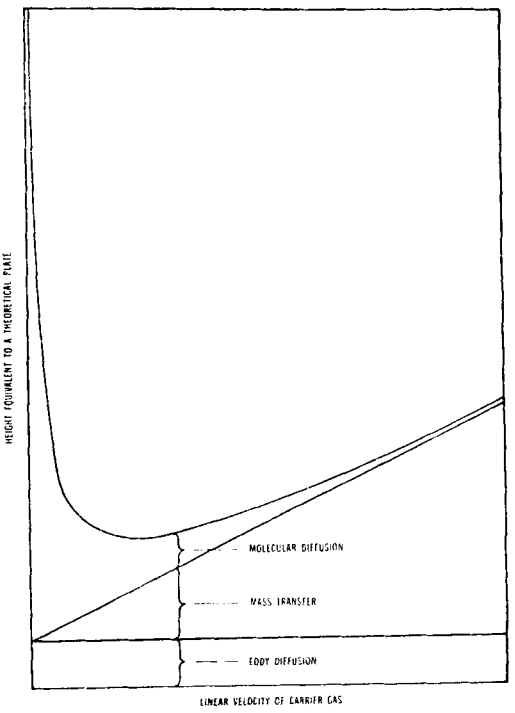

Fig. 1.—Effect of gas velocity on HETP. [After T. Johns (258); courtesy of Beckman Instruments.]

packing, the amount and type of liquid phase (49–51), the size and type of sample (40, 48), the column temperature (49, 50), the length of the column, and the conditions of sample introduction (51, 52). The response time and volume of the detection system are also important considerations (53, 54). Most packed gas chromatographic columns in use today have efficiencies of the order of 500 to 1000 theoretical plates per foot although much greater efficiency can be attained (41, 55). Golay (56) has suggested a "performance index" as a measure of column efficiency and has calculated on a theoretical basis the performance index for an ideal gas chromatographic column. From the difference between this theoretical value and those found in practice, he concludes that there is much room for improvement in basic column design.

Selectivity.—The relative position of the peaks in a gas chromatogram depends upon the relative vapor pressures of the solutes above the liquid phase in the column. The separation of two components in a mixture may, therefore, be expressed as the ratio of the partition coefficients or of the net retention volumes

$$\alpha_{1,2} = \frac{K_2}{K_1} = \frac{\gamma_1^o p_1^o}{\gamma_2^o p_2^o} = \frac{V_{N2}}{V_{N1}} \qquad \text{(Eq. 10)}$$

where α is the relative volatility of substances 1 and 2, γ_1^o and γ_2^o are the activity coefficients, of the solutes in the solvent at infinite dilution, and p_1^o and p_2^o are the vapor pressures of the pure substances.

Where there is little interaction between the solutes and the liquid phase, or where the solute-solvent interactions are of the same magnitude as the interaction between the solute molecules, gas chromatographic separation takes place largely according to the boiling points of the pure substances (57). Such stationary phases are nonselective. Nonpolar liquids, such as saturated hydrocarbons and alkylsilicones, belong to this group. Selectivity increases rapidly with increasing polarity due to the various cohesive forces operating between the solute and the liquid phase. A selective phase is one which will cause separation of substances which have the same boiling points but are of different molecular types (58–60). If $p_1^o = p_2^o$, Eq. 10 becomes

$$\alpha_{1,2} = \frac{\gamma_1^o}{\gamma_2^o} \qquad \text{(Eq. 11)}$$

This ratio has been referred to as the selectivity coefficient (58, 59). Hydrogen bonding plays a very important role in the solute-solvent interaction on polar liquid phases. This was clearly shown by James (61) in the separation of primary, secondary, and tertiary amines. On a nonpolar stationary liquid, the amines emerged in the order of increasing molecular weights, namely in the order of primary, secondary, and tertiary amines. On a very polar column the sequence was reversed, which is the order of increasing hydrogen bonding.

Permanent or induced dipoles in the solute will give rise to interaction with polarizable groups in the solvent and can be used for separation on selective stationary liquids (57). Formation of weak complexes has also been exploited as a means of increasing selectivity (62).

Resolution.—Resolution of closely related compounds in a mixture depends both on the efficiency of the column and on the relative volatility of the solutes in the stationary liquid. The relationship between column efficiency and resolving power has been studied by many workers (32, 55, 63–66).

An important parameter for effective separation is the ratio between the dead volume of the column, V_G^o, and the net retention volume of the solute, V_N. With a reduction in the amount of stationary phase on the column, the plate number often increases while the degree of separation decreases for components with a given relative volatility. This is, at least partly, compensated for by the lower temperature required with lightly loaded columns resulting in increased relative volatility (67). Purnell (55, 63) has defined a separation factor, S, which measures the ability of a chromatographic column to achieve a particular separation

$$S = 36\,[\alpha/(\alpha - 1)]^2 \qquad \text{(Eq. 12)}$$

From S the number of theoretical plates required for separation of two components, for which the relative volatility is known, can be calculated

$$n = 36\,[\alpha/(\alpha - 1)]^2[1 + 2\,(V_G^o/V_N) + (V_G^o/V_N)^2] \qquad \text{(Eq. 13)}$$

Purnell (55) showed that compounds with an α value of 1.15 will be completely separated on a column having 1500 theoretical plates if V_G^o is negligible, whereas, 40,000 plates are required if the ratio V_G^o/V_N is 10.

RECENT ADVANCES IN INSTRUMENTATION AND TECHNIQUES

Detectors.—There has been considerable development in the field of detectors for gas chromatography since James and Martin (4) analyzed eluted acids by a titration procedure. Although the workhorse among detectors for many years has been the katharometer, this de-

tector cell does not possess the sensitivity necessary for the small sample sizes commonly used with high molecular weight compounds and with capillary columns. In 1958, Lovelock (68, 69) described a β-ray ionization detector which was extremely sensitive, and it has since become very popular for gas chromatography in the biochemical field. Argon is used as a carrier gas and is bombarded by α- or β-rays from a radioactive source inside the detector cell. This produces some argon ions and electrons which are accelerated by a high electric field of about 1000 v., reaching velocities sufficient to excite a large number of argon atoms to their metastable state. These excited species normally revert to ground state by emission of electromagnetic radiation. However, if they undergo collision with a molecule having an ionization potential below 11.6 e.v., the metastable argon gives up its excitation energy, thereby causing ionization of the colliding molecule. The ionization current produced in the electric field is amplified and recorded.

Another detector which is extensively used, both because of its high sensitivity, linearity of response, small volume, and simplicity of design is the hydrogen flame ionization detector developed by McWilliam and Dewar (70). The effluent from the column is mixed with hydrogen and fed through a jet where the gas is burned. Ions are formed in the flame by a mechanism of chemi-ionization (71). These are attracted to a collector electrode, thus producing an ionization current which is amplified and recorded.

Other detectors of interest include those based on electron capture (69, 72, 73), thermionic emission (74), and glow discharge (75).

The Column.—From Eq. 4 it is apparent that the retention volume is very much dependent on the amount of stationary liquid in the column. When columns with liquid loadings of the order of 0.5 to 3% are used, instead of the usual 15 to 30%, it becomes possible to decrease substantially retention volumes for compounds of low volatility. By taking advantage of the high sensitivity of the ionization detectors, the sample size can be reduced to such an extent that it will vaporize rapidly into the gas stream at temperatures which are 100 to 200° below the boiling points of the components. At the same time, improvements in instrument design and a greater variety of thermostable stationary phases have extended the range of application to high column temperatures.

An important development in gas chromatographic columns was initiated by Golay (76, 77) who proposed to use capillary tubing coated on the inner surface with the stationary phase instead of packed columns. These capillary columns, which are usually made from stainless steel, glass, or nylon tubing, range in length from 50 to several hundred feet. Efficiencies approaching a million theoretical plates have been achieved, but this is not directly comparable with the efficiencies of packed columns. The performance of coated capillary columns with respect to speed, column efficiency, and resolution has been thoroughly studied by Desty and co-workers (78–80). Purnell (55, 63) has shown that analyses performed with very long capillary columns of up to one-half million plates can be closely reproduced by a 7-ft. packed column of 5000 plates in about one-hundredth the time. Capillary columns have very low capacities and rarely operate efficiently if the quantity of any single component to be separated exceeds 10^{-7} Gm. A sample splitting device between the injection port and the column is, therefore, essential. There is still considerable controversy regarding the relative merits of packed columns and capillary columns. Undoubtedly, both types have their strengths and weaknesses. Although capillary columns have gained considerable popularity for separation of closely related compounds, especially in the petrochemical field, it is unlikely that they will replace the packed columns completely for analytical gas chromatography.

Tailing.—When polar materials are gas chromatographed on a nonpolar liquid phase, tailing can often cause considerable problems. It will distort the symmetry of the peaks, interfere with clean-cut separation, and make quantitative analysis very difficult. This is especially true if the concentration of liquid phase is low. The reason for this tailing effect is that most solid support materials are not completely inert, but contain active spots which cause adsorption. When adsorption takes place, the retention time is much influenced by the sample size. Large samples cause an earlier appearance of the front accompanied by a peak broadening. This has been attributed to a Langmuir adsorption isotherm which has reached the saturation limit (81). The effects of the solid support material on the retention volumes have been studied by Scholz and Brandt (82), Bens (83), Weinstein (84), and others. Adsorptive effects are due to weak van der Waals forces and strong hydrogen bonding forces, caused mainly by the presence of free hydroxyl groups on the support material. Several methods of deactivation have, therefore, aimed at removing the hydroxyl groups by reaction with dichlorodimethyl silane (85, 86), hexamethyldisilazane (81, 82), or by methylation (*cf.* 82).

Ormerod and Scott (87) have reported that silver coating of firebrick drastically reduces adsorptive effects. Others have added small amounts of fatty acids (4, 88), their salts (89), or various surface-active agents (81, 90, 91). These tend to deactivate hydrogen bonding sites, spread the stationary phase over the surface of the support, and possibly act as a "glue" to span the interface between the liquid and the solid (91). Addition of small amounts of a very polar stationary liquid to a nonpolar liquid will markedly reduce tailing without changing the retention values (81, 88, 92).

Temperature Programing.—Because of the band broadening factors expressed in the van Deemter equation, the peaks of a normal isothermal gas chromatogram become wider and lower as the retention time increases. Furthermore, it is possible for high boiling components to go undetected because of very long retention times (93–95). By increasing the temperature during the run of a chromatogram, it is possible to separate rapidly a mixture of components boiling over a wide range of temperature, and all solutes emerge as sharp, well-defined peaks of nearly the same shape (Fig. 2). With a linear temperature rise, each solute is eluted at a characteristic retention volume in the same way as for isothermal gas chromatography (96). The theoretical aspects of temperature-programed gas chromatography have been discussed by Giddings (97) and Said (98). Many commercial instruments have incorporated the feature of temperature programing. Dual column systems are sometimes used in order to maintain a stable base line by canceling the effects of increased column bleeding at higher temperatures (99).

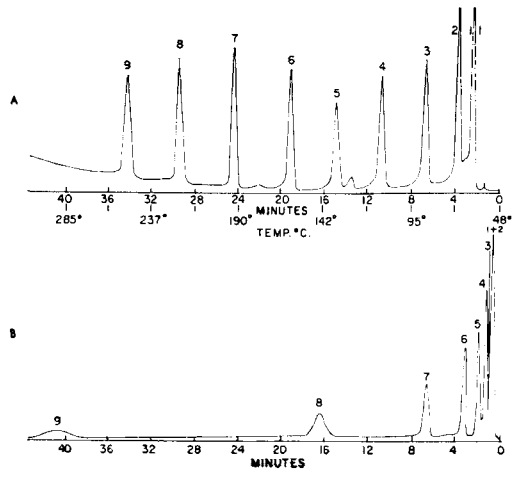

Fig. 2.—Gas chromatography of alcohols. A, temperature programing; B, isothermal gas chromatography at 165° of (*1*) methanol, (*2*) ethanol, (*3*) 1-propanol, (*4*) 1-butanol, (*5*) 1-pentanol, (*6*) cyclohexanol, (*7*) 1-octanol, (*8*) 1-decanol, and (*9*) 1-dodecanol. [After S. Dal Nogare and C. E. Bennett (93); courtesy of *Analytical Chemistry*.]

Sample Pyrolysis.—Organic substances, upon heating, undergo various decomposition reactions, the mechanism of which depends upon the structure of the molecules. Although the pyrolysis reactions are often extremely complex, they are reproducible under constant conditions. Pyrolysis has been used in conjunction with gas chromatography by several investigators (100–111) both for qualitative and quantitative analysis. The principle is simple. The product to be studied is applied to a filament which is placed in the stream of carrier gas. The filament is heated at a predetermined temperature for a few seconds by means of an electric current, and the pyrolysis products are carried by the gas stream to the column for separation. The method is particularly suited for analysis of polymers which, at a certain temperature, depolymerize to give the original monomers (111). However, numerous other compounds have been analyzed by this method, including amino acids, barbiturates, alkaloids, and steroids.

APPLICATIONS

Qualitative Analysis.—Identification of components in a mixture by gas chromatography is most frequently based on retention data which are related to the R_f value commonly used in paper chromatography. A comparison of the retention volumes (or times) of the unknown constituents with those of known compounds will often suffice to identify the former. However, the retention volumes are dependent on experimental conditions, many of which cannot be easily reproduced. It has, therefore, been recommended that retention data be reported in absolute units, such as specific retention volumes or partition coefficients. Somewhat simpler, from an experimental point of view, is the use of relative retention volumes (or times), where the effects of operating parameters are considerably reduced, depending mainly on the nature of the stationary phase and on the column temperature. The relative retention values are expressed by

$$r_{1,2} = \frac{V_{N1}}{V_{N2}} = \frac{V_{g1}}{V_{g2}} = \frac{V'_{R1}}{V'_{R2}} = \frac{t_1}{t_2} \quad \text{(Eq. 14)}$$

where the subscripts 1 and 2 stand for the sample component and the standard, respectively, V'_R is the net uncorrected retention volume, and t is the net retention time measured from the air peak to the sample peak.

Several modifications of this principle have been proposed. Evans and Smith (112), using a primary standard (*n*-nonane) and one or more internal standards, express the retention of the unknown relative to that of *n*-nonane as follows

$$R_{X9} = R_{XN} \cdot R_{N9} \qquad \text{(Eq. 15)}$$

where R_{X9} is the retention of the unknown relative to *n*-nonane, R_{XN} is its retention relative to the internal standard, and R_{N9} is the retention of the internal standard relative to *n*-nonane. Two internal standards are used whenever possible, one emerging on either side of the unknown, and the mean R_{X9} value is determined.

A closely related method is used by Kovats (113–116), who calculates a "retention index" on the basis of two reference standards.

The logarithm of the net retention volume of a substance is proportional to the free energy of solution of the solute molecules in the stationary phase. The free energy of solution is an approximately additive function of the groups constituting the molecule. Therefore, if the logarithm of the net retention volume (or relative retention volume) is plotted against some increasing property, such as the number of carbon atoms in a homologous series, a straight line is obtained. The slope of this line depends on the nature of the stationary phase (61, 117, 118).

In a complex mixture, there is always the possibility of two or more substances having the same retention characteristics on a particular column. This uncertainty with respect to separation and identification can be greatly reduced by making use of two or more columns of different polarity. Many methods are based on this principle. Lewis, Patton, and Kaye (119) used homologous series plots in which the logarithms of the elution times obtained on a nonpolar column were plotted against those obtained on a polar column. James (25) used log plots of the relative retention volumes for two stationary phases as a means of characterizing fatty acids. Brown (120) proposed a three-column method, basing the identification of the components of brown coal tar on the retention data measured with three different types of stationary liquids: a nonpolar liquid, an electron donor, and an electron acceptor. He defined an "affinity fraction," A, of the substance to be identified for each phase as

$$A_1 = r_1/(r_1 + r_2 + r_3)$$

where r_1, r_2, and r_3 are the retention volumes (or times) on the three stationary liquids under controlled conditions. The affinity fractions of a given substance in the three liquids were plotted on a triangular graph.

Because of the efforts required to achieve positive identification of complex mixtures solely on the basis of retention characteristics, many investigators have preferred to use gas chromatography in combination with chemical reactions or auxilliary instrumentation.

Walsh and Merritt (121) used a combination of retention volume and functional group analysis for the identification of chromatographic peaks. The functional group classification was accomplished by means of a stream splitting device attached to the exit tube from a thermal conductivity cell. As each chromatographic peak passed through the detector, the gas stream was split and the vapor collected in a set of reagents. A similar procedure was used by Dubois and Monkman (122). Various other chemical reactions, both before and after chromatographic separation, have been proposed for identification purposes (123, 124).

The combination of a gas chromatograph and a time-of-flight mass spectrometer is extremely useful for rapid and positive identification of complex mixtures. The mass spectra of the components can be scanned and recorded simultaneously with the running of the chromatograph (125, 126). The input system to the spectrometer can be designed so as to trap the sample from the column, letting it leak into the spectrometer long enough to allow the analog output system to record the spectrum.

Identification of gas chromatographic peaks by means of infrared or ultraviolet spectrophotometry is also extensively used (127–134). The fractions may be collected in a solvent or in solid potassium bromide and the appropriate analyses made, or a continuous scanning of the gaseous eluate may be carried out.

Another means of identification which is likely to become increasingly important is to use a combination of a selective and a nonselective detector. The electron capture detector is a selective detector having a high degree of specificity for halogenated compounds as well as for conjugated carbonyls and organometals (72, 135). Hydrocarbons, alcohols, amines, ketones, etc., produce virtually no response. Varadi (136) has developed an ionization detector which gives both qualitative and quantitative information. It is composed of a thermionic ionization detector unit for quantitative determination combined with a radio-frequency m/e analyzer, where the characteristic mass number of the gas component can be

determined. The signals from both detector parts are amplified and recorded on separate recorders.

Quantitative Analysis.—With a differential detector in combination with a potentiometric strip chart recorder, the substances appear as peaks having the dimensions of (millivolts) (time). The areas of these peaks are related to the concentration of component vapor in the carrier gas. The factor for converting peak area into weight varies with the detection system, the carrier gas, and the component to be determined. For accurate work, the detector response must be linear over a reasonable range of vapor concentration in the carrier gas. Accuracy and precision also depend on the sensitivity and stability of the detector and amplifier, on the method of sample application, and, in the case of capillary columns, on the linearity of the stream splitting system. Gas samples can be applied to the column with great accuracy by means of a gas sampling valve. For liquids and solids the problem is not nearly as simple, and many attempts have been made to develop reproducible sampling techniques. With the small samples required for very high boiling materials, especially in conjunction with ionization detectors and capillary columns, a simple syringe technique is commonly used although the sample size cannot be accurately reproduced. This usually requires an internal standard for calibration. A known amount of a reference substance is added to the sample and the mixture chromatographed. The composition of the sample is obtained from a standard curve based on chromatograms of several accurately known mixtures of the standard and the sample components. Two or more internal standards should be used if the sample contains substances with a wide range of boiling points (137).

Another method frequently used is referred to as internal normalization. If the detector response is approximately equal for all the components in a mixture, the total area under all the peaks is normalized to 100% and each peak calculated as a percentage of the total. If the detector response is markedly different for the different constituents in the sample, and this is usually the case, correction factors must be applied to each peak before they are added and proportioned.

Various methods have been proposed for the measurement of peak area. The simplest method which still gives reasonably good results when the peaks are symmetrical and well separated is triangulation, in which the area is assumed to be equal to the area of the triangle formed by the tangents through the points of inflection and the peak base. Greater precision can be achieved by means of a planimeter, although measurement errors for small peaks can be considerable. Besides, the method is slow and subject to operator fatigue. Based on the fact that gas chromatographic peaks, in the absence of adsorptive effects, closely approximate a Gaussian distribution curve, Bartlet and Smith (138) calculated peak areas from the peak height and the standard deviation for the curve by the formula

$$A = h\sigma\sqrt{2\pi} = 2.507\ h\sigma \qquad \text{(Eq. 16)}$$

where A is the area, h is the peak height, and σ the standard deviation for the normal distribution curve corresponding to the peak (= one-half peak width at 0.607 h).

The most accurate estimation of peak area may be obtained by a number of integrators based on analog or digital computation. A widely used type of analog computor is the ball and disk integrator which is relatively simple and inexpensive. It has an inherent accuracy within ±0.1% of full scale, and with proper care and operation a high degree of precision may be achieved (139). Recorder-integrator errors in gas chromatography area measurements have been studied by Orr (140). A formula has been developed to correct for these errors. With careful work, quantitative analysis by means of gas chromatography can give a high degree of precision and accuracy. This has been shown by many workers, but perhaps most clearly by Evans and Scott (141). Based on 110 consecutive determinations, the overall spread was 1%, the coefficient of variation 0.23%, and the 95% confidence limits ±0.35%. This compares very favorably with most instrumental methods of analysis.

Although gas chromatographic columns should be chosen so as to produce optimal separation, a complete resolution cannot always be obtained. Quantitative analysis may be made even if the peaks are overlapping. The problems involved and the methods used to overcome them have been discussed by several investigators (138, 142, 143).

ANALYSIS OF COMPOUNDS OF PHARMACEUTICAL INTEREST

It is only natural that the early applications of gas chromatography to pharmaceutical analysis have been concerned with very volatile

materials, such as anesthetic gases (144), alcohols, and other solvents, including water (145–149), nitroglycerin (145), essential oils, and their constituents (145, 150–155). In fact, gas chromatography has been a great impetus to the study of essential oils and perfumes, as well as the flavor and aroma substances present in food and beverages (156, 157). Because of the complex nature of these materials, no satisfactory method for a thorough, objective analysis was available until gas chromatography was developed. It is now possible, in a short time, to identify a volatile oil on the basis of the retention data and peak ratios of the chromatogram, and the components can be determined quantitatively. The method can also be used to detect adulterations and even to determine the geographical origin of the oil (158).

Gas chromatography has been used for monitoring the concentration of anesthetics in the patient's breath during the course of an operation (159) and to determine the absorption, distribution, and elimination of diethyl ether (160–162). Ether and chloroform, as well as various essential oil constituents, have been determined quantitatively in a variety of pharmaceutical preparations (150, 152, 163, 164).

Gas chromatography was developed in connection with the separation of organic acids, and a tremendous amount of work has been done in biochemical research involving studies of fatty acids and lipids and their metabolism in man and animals. The fatty acids are usually converted to the methyl esters by one of several methods (165) although the free acids may also be gas chromatographed (4, 166–168). Various polyesters are particularly selective for separation and identification of fatty acid esters (169, 170). The specific distribution of fatty acids in the glycerides of vegetable fats has been studied by means of gas chromatography (171) which promises to be an exceptionally useful method for quality control of fats and oils, including identification, detection of adulteration, and misbranding (172), and determination of the amounts of free fatty acids present (173). More recently it has been shown that the triglycerides may be gas chromatographed directly (174–176). Whether this method will prove to be more valuable than the standard procedure based on the fatty acid methyl esters still remains to be seen.

The resin components of marihuana (*Cannabis sativa*) have been separated by Kingston and Kirk (177) and by Farmilo and Davis (178). In addition to the major component, cannabinol, a large number of smaller peaks illustrate the complex nature of this material. The method appears to be of value for determination of the origin of seized marihuana.

Although aliphatic amines were among the first substances gas chromatographed by James (61), it is only recently that the method has been applied to pharmaceutically important amines. Brochmann-Hanssen and Svendsen (179, 180) have gas chromatographed a number of commonly used sympathomimetic amines on silicone rubber SE-30 and achieved excellent separation in most instances. The reaction between amines and ketones is useful for identification purposes and to separate certain isomers, such as ephedrine and pseudoephedrine. The catechol amines, epinephrine and norepinephrine, have been gas chromatographed after treatment of the triacetyl derivatives with hexamethyldisilazane.

Fales and Pisano (181) have also separated a number of biologically important amines including histamine, seratonine, tyramine, and some sympathomimetics.

In a recent publication Parker, Fontan, and Kirk (182) reported gas chromatographic separation and identification of a large number of tranquilizers, including phenothiazine derivatives, diphenylmethane derivatives, butanediols, propanediols, and others. No peaks were observed after application of rauwolfia alkaloids. Occasionally, following storage of some of the tranquilizers, new small secondary peaks appeared which increased in height with the passage of time. This suggested a possible application of the method to quality control during manufacture.

Gas chromatography of phenothiazine derivatives has also been reported by Anders and Mannering (183).

During the past year, the barbiturates and some related substances have been successfully gas chromatographed on various stationary liquids in low concentration (184–186). Not all compounds can be separated on the same column, but by using two columns, one with a nonpolar phase such as Apiezon L and one with a moderately polar polyester, a large number of possible combinations can easily be separated. The method has been used for identification of pharmaceutical preparations and for the study of the metabolism and excretion of barbiturates in humans (186, 187). The partial transformation of phenobarbital to *p*-hydroxyphenobarbital, and the demethylation of N-methyl substituted barbituric acid derivatives have been confirmed

by means of gas chromatography.

Stainier and Gloesener (188) have analyzed certain ester alkaloids and related synthetic drugs by hydrolysis and gas chromatography of the liberated alcohols. However, such pretreatment is usually not necessary. The volatile tobacco alkaloids can easily be gas chromatographed, as was shown by Quin (189–191) in 1958. More surprising is the fact that many alkaloids of high molecular weights and relatively low stability can be gas chromatographed as such. This was first demonstrated by Lloyd, *et al.* (192), who gas chromatographed 45 different alkaloids on low-loaded columns of silicone rubber SE-30. In this way, strychnine was separated from brucine, quinine from cinchonine, and the various major alkaloids in opium were separated from one another. Eddy, *et al.* (193), used this method for the determination of the geographical origin of opium. Brochmann-Hanssen and Svendsen (194) also applied gas chromatography to the separation and study of alkaloids in several crude drugs. Alkaloidal salts were gas chromatographed directly without prior isolation of the base. The salts dissociated in the flash heater which was maintained at about 325°, and the alkaloids were eluted as the free bases. The decompositions sometimes observed when certain alkaloids were subjected to gas chromatography were discussed (194).

Few classes of drugs are of greater importance than the steroids. Therefore, when it was shown in 1960 (195, 196) that steroids could be analyzed by gas chromatography with little or no decomposition, this created a tremendous interest among biochemists and pharmaceutical chemists. A new tool had become available for research in this area, and during the last two years an impressive amount of work has been done with it, particularly by Horning and his co-workers. Silicone rubber SE-30, the more polar and more selective fluorosilicone fluid QF 1-0065 (197), and various heat-stable polyesters (198, 199) have been found to be satisfactory stationary phases for gas chromatography of steroids. The method has been applied to corticosteroids (200, 201), androgens (199, 202), estrogens (202, 203), steroidal amines (204), sapogenins (205), cholesterol (86, 196), bile acids (86, 202), vitamin D (207), etc.

Synthetic estrogens, such as diethylstilbestrol, have been estimated in extracts of tissue, feces, and urine (208).

Several attempts have been made over the years to separate mixtures of amino acids by gas chromatography. The amino acids are not sufficiently volatile to be gas chromatographed directly, but may be converted to volatile products by various reactions. These include oxidation to aldehydes (209, 210), reaction of the amino group with acetic anhydride or trifluoroacetic anhydride followed by esterification of the carboxyl group (211–215), or treatment with hexamethyldisilazane (216). Pisano, *et al.* (217), have gas chromatographed the phenylthiohydantoin and the dinitrophenyl derivatives of amino acids.

The best results so far appear to have been obtained by Johnson, *et al.* (215, 218), who have separated a large number of amino acids as the *n*-amyl esters of the N-acetylamino acids. The method has been applied to hydrolysates of peptides and proteins (218).

Carbohydrates, including mono-, di-, and trisaccharides, as well as simple glycosides, have been gas chromatographed, either as the methyl ethers (219–223), the acetates (224–226), or the trimethylsilyl ethers (227). Using a column of fluorosilicone fluid QF 1-0065, Horning, *et al.* (226), separated the tetraacetates of α- and β-glucose. No separation was achieved with the less selective silicone rubber SE-30.

SPECIAL PROBLEMS IN PHARMACEUTICAL ANALYSIS

Determination of Purity.—An important aspect of pharmaceutical analysis is concerned with the identification and determination of trace impurities, and gas chromatography is excellently suited for this purpose. Bennett, *et al.* (228), have determined trace amounts of impurities in various solvents with a reproducibility of better than 2% for well-resolved peaks. Figure 3 illustrates the importance of instrument sensitivity. Chromatograms I and II are typical runs obtained with conventional equipment. Runs III and IV were made at a one hundred-fold increase in sensitivity. The total concentration of impurities in the cyclohexane sample was 0.3% and could not be detected by infrared analysis. Maruyama and Seno (229) determined trace amounts of methanol in ethanol and reported a detection limit of 0.005%. Impurities in chloroform have been studied by Gloesener (230). An interesting report by Iguchi, *et al.* (231), describes the application of gas chromatography to the determination of trace amounts of arsenic. The sample is reduced with zinc and acid, and the arsine is gas chromatographed with hydrogen as the carrier gas. As little as 1 mcg. could be detected, while the limit for quantitative determination was 4 mcg.

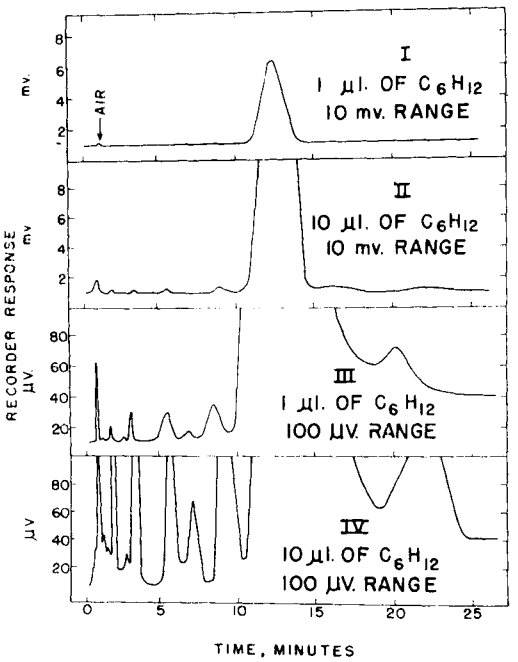

Fig. 3.—Chromatograms of cyclohexane at 10 mv. and 100-µv. full-scale sensitivity ranges. [After C. E. Bennett, *et al.* (228); courtesy of *Analytical Chemistry*.]

Using a column of toluene on granular Teflon and a temperature of −78°, Bergmann and Martin (232) separated bromide and chloride as the hydrogen halides. The sample was added to 70% sulfuric acid, whereby the halide ions were converted to the covalent hydrogen halides which were collected in a nitrogen trap and then gas chromatographed. Concentrations down to 0.01 p.p.m. in water could be detected. The method also permitted easy determination of trace amounts of chloride in bromide, a difficult analysis by other methods.

Much work has been done in the field of pesticide residues on fruits and vegetables. The electron capture detector is excellent for analysis of chlorinated pesticides because it permits determination of submicrogram amounts of the pesticides in the presence of a considerable excess of coextracted material (233). Coulson, *et al.* (234), used a coulometric detection system for quantitative analysis of halogenated pesticide residues.

A concentration technique is frequently needed in order to eliminate interference from other components in the sample (235, 236). Hornstein, *et al.* (173), determined the free fatty acids in fat emulsions by adsorption on an anionic exchange resin, esterification of the acids on the resin by means of methanol and hydrochloric acid, and gas chromatography of the isolated methyl esters. Trace components in an essential oil were determined after all polar matrix compounds had been removed by adsorption on silica gel (237).

Elemental Analysis.—Rapid methods have been developed for determination of carbon and hydrogen by means of gas-solid chromatography (238, 239). The organic sample is burned in a stream of oxygen, and the resulting carbon dioxide and water vapor are passed through a calcium carbide tube which converts the water to acetylene. The gases are concentrated in a freeze trap and then vaporized and swept into the chromatographic system by the carrier gas. The method is claimed to be very precise (0.5% for carbon and 0.1% for hydrogen) and more rapid than conventional methods. The time of analysis is about 20 minutes for a single determination and about 10 minutes each for a continuous series of analyses including area measurements (238). Vogel and Quattrone (240) have modified the method somewhat. Using gas-liquid chromatography with dodecyl phthalate as the stationary liquid and oxygen as the carrier gas, they eliminated the need for isolating the products of combustion from the combustion atmosphere.

Direct gas chromatographic analysis of oxygen in organic compounds has been reported by Goetz (241) and by Suchanec (242). Oxygen is converted to carbon monoxide at high temperatures by means of charcoal (241) or a platinum-carbon mixture (242). Suchanec reports a standard deviation of ±0.17%. Gas chromatographic methods are also available for determination of organically bound nitrogen (243) and sulfur (244).

Determination of Surface Area.—Surface area and particle size determinations are often of considerable importance in studies related to formulation of pharmaceutical dosage forms. This is a field in which gas chromatography has been particularly useful. In 1958 Nelsen and Eggertsen (245) described a method for determination of surface area based on adsorption chromatography.

The column packing is the sample whose surface area is to be determined and the mobile gas is a mixture of a suitable adsorbate (nitrogen) and an inert gas (helium). A known mixture of nitrogen and helium is passed through the sample in a U-shaped tube, the effluent being monitored by a thermal conductivity detection system. When a stable base line is obtained, the sample tube is immersed in liquid nitrogen. The sudden cooling causes absorption of nitrogen

and a corresponding negative peak on the recorder chart. After the equilibrium condition is established again, as indicated by the return of the recorder pen to the base line, the coolant is removed, causing desorption of nitrogen from the sample and a positive peak on the chart. Both peak areas are of the same size, being a function of the amount of nitrogen absorbed by the sample, and may be used for surface area measurements by either an absolute or a comparative method. Modifications of this method have been described by Roth and Ellwood (246) and by Stock (247).

Separation of Isomers.—A problem which has intrigued pharmaceutical chemists for a long time is the separation of isomers, particularly optical isomers.

Partial separation of racemic mixtures has been reported by means of an optically active stationary liquid such as *d*-tartaric acid ethyl ester (248). However, best results seem to have been achieved so far by converting the optical isomers to diastereomeric derivatives prior to the gas chromatographic analysis. In this way, Casanova and Corey (249) separated the optical isomers of camphor as ketals prepared from D(-)-2,3-butanediol. The choice of liquid stationary phase was very critical, as was also shown in the separation of the four stereoisomeric racemates of menthol. Using Hyprose SP-80 [octakis (2-hydroxypropyl) sucrose] and a 12-ft. column, Moore and Kossay (250) were able to determine quantitatively menthol, isomenthol, neomenthol, and neoisomenthol obtained by catalytic hydrogenation of thymol. The amounts of the various isomers in peppermint oil can also be determined (158, 251). Optical isomers are not separated by this procedure.

Bates, *et al.* (252), have isolated and characterized the four stereoisomeric farnesols on a 5-ft. column containing 20% of polyethylene glycol 20M. The isomers of ionone and methylionone have likewise been separated by gas chromatography (253, 254). One may expect considerable work in this field in the future, particularly in regard to high molecular weight drugs.

Process Control.—Chemical analyses for process control must be rapid and dependable, and the instrumentation must be rugged and preferably suitable for automatic operation. Gas chromatography satisfies most of these requirements, and during the last few years it has become an important tool for monitoring chemical reactions. The method is not continuous in the strict sense, since the separation does require a definite time. However, it can be made automatic in that it is carried out according to a predetermined schedule without manual operation. The instrument itself samples the stream, passes the sample through the column, and records the components as they emerge (255–260). High speed chromatography is especially important for this type of application. Several commercial instruments for process analysis are available.

Miscellaneous Applications.—Gas chromatography has been a valuable aid in the determination of structures of organic molecules, particularly in conjunction with pyrolysis or other degradation reactions. Formyl and acetyl groups in digitalis glycosides have been identified by gas chromatography after transesterification with methanol (261, 262). Lloyd, *et al.* (263), used the method to determine the position of aromatic methoxyl groups in alkaloids related to powelline, while Cox (264) identified the side chains of steroids by pyrolysis followed by gas chromatography. Others have been able to use gas chromatographic techniques for determination of the amino acid sequence in proteins (265) and the arrangement of fatty acids in triglycerides (171). Dhont (108) has made a systematic study of the correlation between structure and the pyrolysis products of organic compounds.

Because of its speed and selectivity, the gas chromatographic method is well suited for the study of reaction mechanisms and reaction kinetics, both with regard to the synthesis of drugs, their *in vitro* deterioration (266), and their biotransformation. The effects of experimental parameters on the yield of a synthetic procedure may be readily evaluated and optimum conditions established.

CONCLUSIONS

No attempt has been made to exhaust completely the existing literature with reference to the applications of gas chromatography to pharmaceutical and related analysis. However, it should be apparent that the method has become a powerful tool for research as well as routine analytical work. The use of gas chromatography for high molecular weight compounds of low volatility, a very recent adaptation, has greatly stimulated interest among pharmaceutical chemists, since the vast majority of drugs fall in this category. It is important to realize that many chemical substances which are quite unstable even at

room temperature in the presence of air and moisture, frequently hold up very well in the vapor state in the inert atmosphere of a gas chromatographic column. Where the substance to be analyzed is unstable at the elevated temperature required for volatilization, stable, volatile derivatives can often be prepared, or the pyrolysis technique may be applied.

The pharmaceutical industry is making use of gas chromatography on a rapidly increasing scale and it is not unreasonable to assume that the method may be recognized by the official compendia in the near future.

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Research Articles

Substituted Heterocyclic Thioureas I

Antitubercular Activity

By ARTHUR C. GLASSER and RICHARD M. DOUGHTY

A series of substituted 2-pyridyl and 4-(1-phenyl-2,3-dimethyl)-5-pyrazolone thioureas have been synthesized and tested for their antitubercular activity against *Mycobacterium tuberculosis* in Dubos medium. The minimum inhibitory concentrations of the compounds studied ranged from 10 mg. per 100 ml. to 0.16 mg. per 100 ml.

THIOUREA and its simple derivatives have been shown to have a limited tuberculostatic activity (1). *p*-Aminophenyl alkyl ethers also have a definite inhibitory effect (2), and when the two classes of compounds are combined through N-substituted thioureas this activity is enhanced (3–7). The tuberculostatic activity of the thioureas has been reviewed by Schroeder (8). This study is concerned with the influence on tuberculostatic activity by the substitution of the thiourea molecule with heterocyclic rings such as 2-pyridyl- or 4-(1-phenyl-2, 3-dimethyl)-5-pyrazolone in conjunction with alkyl or *p*-alkoxyphenyl substitution. The 1-*p*-alkoxy-

Received March 19, 1962, from the College of Pharmacy, University of Kentucky, Lexington.
Accepted for publication April 23, 1962.
This investigation was supported in part by research grant E-4089, National Institutes of Health, Public Health Service.