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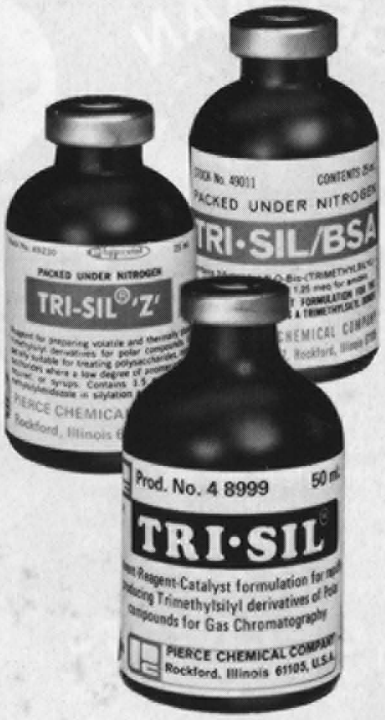

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CHREV. 78

IDENTIFICATION OF GAS CHROMATOGRAPHIC ZONES IN PRACTICAL GAS–LIQUID CHROMATOGRAPHY

INFLUENCE OF ADSORPTION ON RELATIVE RETENTION

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(Received May 8th, 1974)

Gas chromatographic identification methods based on the comparison of experimentally determined retention values of chromatographed zones of unknown compounds with the corresponding values for zones of known compounds are widely used[1]. The use of this simple technique for comparing retention values is limited, however, by the inadequate inter-laboratory reproducibility of retention values. Inter-laboratory non-reproducibility of retention values can apparently be attributed to the non-uniform properties of the sorbents prepared by different workers using different procedures and solid supports of different quality. It should be noted that the differences in the properties of the sorbents in gas–liquid chromatography (GLC) have not been given sufficient acknowledgement in the literature as being the cause of inter-laboratory non-reproducibility of retention values, although the practical value of the chromatographic technique is due largely to the possibility of its wide application in many laboratories.

The advances achieved in recent years in the development of the retention theory and in investigating the properties of sorbents in GLC have helped to explain the non-reproducibility of retention values and to develop new methods for identifying chromatographic zones.

In the modern theory of GLC, the retention of compounds chromatographed is considered, taking into account the real distribution of the liquid stationary phase (LSP) on the solid support[1–12]. Investigations conducted by various methods indicate, in general, that the LSP distribution is complex. At first the LSP fills mainly the narrow pores of the solid support, then the pores of larger diameter, and the LSP film on the walls of the wide macropores grows thicker. With an LSP content on ordinary solid supports exceeding 1–3% and with good wettability, a continuous LSP film apparently forms on the surface of the solid support; when the surface wettability of the solid support is low, the liquid phase can be observed on the surface in the form of separate insular drops. The nature of the distribution also depends greatly on the technique of applying the LSP, the conditions of the subsequent aging of the sorbent[12], etc. In practical gas–liquid chromatography, according to various workers[2–9], the model of a sorbent obtained on the basis of ordinary diatomaceous supports with an LSP content of more than 1–3% can be regarded as a porous solid (solid support) covered with a continuous LSP film of variable thickness (see Fig. 1). This model agrees well with the following different experimental data: the dependence

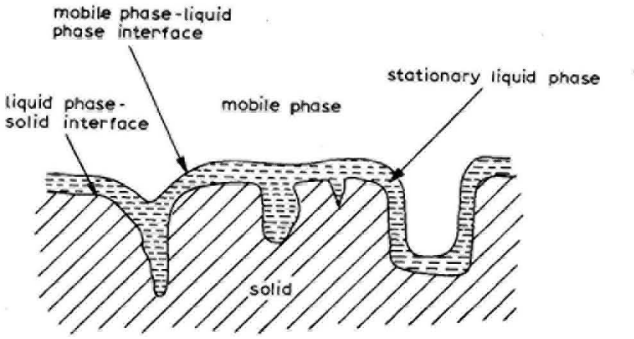

Fig. 1. Model of sorbent in gas–liquid chromatography.

of the height equivalent to a theoretical plate on the flow velocity of the carrier gas[10]; the variation in pore distribution in relation to the sorbent surface with an increase in LSP content on the solid support[5,9,11]; the dependence of retention in the vicinity of the melting point of the LSP[6]; and the results of special investigations of the support with the use of a marked surface[7].

Therefore, in considering the retention of volatile compounds, we have henceforth adopted the model of complete or quasi-complete coverage of the surface of a solid support with an LSP film.

In agreement with the above, a sorbent in GLC cannot be regarded solely as an LSP. At the LSP–gas support interface there is one surface phase (gas–liquid) and at the LSP–solid support interface another surface phase (liquid–solid). The properties of the LSP in a macrofilm on a solid support usually coincide with the properties of the bulk phase of the LSP. In the case of thin films (several molecular layers), it is necessary to take into account the effect of the field of the solid support. Thus, a sorbent in GLC is a polyphase sorbent. The development of the equilibrium theory of the retention of volatile compounds is associated with the contribution of the retentions on the separate phases of a sorbent to the total retention volume[5,13-17]. The retention value of a volatile substance is determined by its interaction with at least one bulk and two surface phases, namely the dissolution of the substance chromatographed in the LSP and its adsorption on the gas–LSP and LSP–solid support interfaces. For this case, the net retention volume (V_N) can be represented by the following equation within the framework of the theory of equilibrium chromatography under the conditions of linear isotherms of dissolution and adsorption[15]:

$$V_N = K_l v_l + K_{gl} S_l + K_l K_S S_S \qquad (1)$$

where K_l is the distribution constant of the chromatographed compound in the gas–LSP system, K_{gl} is the adsorption constant of the chromatographed compound in the gas–LSP surface system, K_S is the adsorption constant of the chromatographed compound in the LSP–solid support system, v_l is the volume of LSP in the column, S_l is the total surface area of the LSP at the LSP–gas interface, and S_S is the total surface area of the LSP–solid support interface.

In the general case, when in a sorbent the number of phases capable of retain-

ing volatile substances exceeds the above three phases, the following generalized equation is valid[15]:

$$V_N = \sum_i^n \frac{\partial V_N}{\partial v_i} \cdot v_i + \sum_j^m \frac{\partial V_N}{\partial S_j} = \sum_i^n K_i v_i + \sum_j^m K_j S_j \qquad (2)$$

where v_i is the volume of the i-type LSP characterized by an effective distribution coefficient $K_i = \partial V_N / \partial v_i$ (LSP types: LSP in micropores, LSP in the macrolayer, etc.), S_j is the area of a j-type surface characterized by an effective distribution coefficient K_j (surface types: the surface of an uncovered support, the surface of an LSP macrolayer, the surface of an LSP monolayer, and so on).

As a particular case, it is possible to obtain, from eqn. 1 and also from the equation for the retention volume in classical GLC[13], the equation of Martin[16], the equation for the retention volume in gas–adsorption chromatography for macroporous adsorbents[18], etc. It should be stressed that in the general case, practical utilization of Fig. 2 is complicated as it requires the use of additional quantitative data on the distribution of the LSP over the surface of the solid support, *i.e.*, on the quantitative phase characteristics of the adsorbent used. Therefore, in practice, a simplified form of eqn. 1 is usually applied, which describes the retention on a sorbent obtained with complete coverage of a solid support with an LSP macrofilm. This equation has been used successfully in analyzing adsorption phenomena and determining the distribution and adsorption constants in GLC by a number of investigators, such as Conder *et al.*[14], Urone *et al.*[19], Gritchina and Dreving[20], Liao and Mortire[21], the present author and co-workers, and by other workers. It was shown that the contribution of adsorption of the compounds chromatographed to the retention volume in GLC often has a considerable value which cannot be neglected.

The adsorption of volatile substances to be analyzed on the surface phases

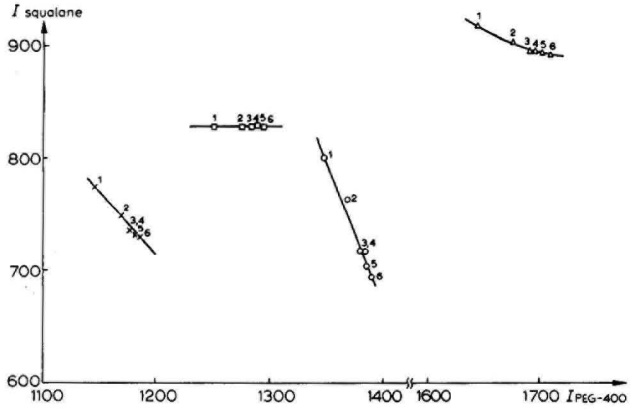

Fig. 2. Dependence of retention indices for series of compounds on squalane as a function of retention indices of the same compounds on PEG-400 with the use of different solid supports (according to the data of Evans and Smith[32]). Compounds: △, benzonitrile; ×, 2-hexanone; □, chlorobenzene; ○, 1-pentanol. Supports: 1, Chromosorb W; 2, Celite; 3, Anachrom; 4, Chromosorb G; 5, Gas-Chrom; 6, Chromosorb G, silanized.

of the sorbent must also exert an effect upon the relative retention values[5] that are used in identifying chromatographic zones.

Various relative retention values are used for identification[1]. Known relative retention values can be represented as particular cases of the expression[22]

$$R = P\left(k + \frac{r_i - r_m}{r_n - r_m}\right) = P\left(k + \frac{\Delta r_{im}}{\Delta r_{nm}}\right) \tag{3}$$

where R is the relative retention value in a given coordinate system, P and k are constants for a given coordinate system, r_i, r_m and r_n are the corrected (net) retention values of the ith compound and the mth and nth standard substances (or their functions) and Δr_{im} and Δr_{nm} are the differences of the corrected retention values (or of their functions).

In gas chromatography, for determinations under isothermal conditions, it has been recommended that one should use the relative retention value[23,24], the retention factor[25–27] and the arithmetic factor[28], which is also used for measurements in temperature programming[29]. A characteristic similar to the arithmetic factor was proposed by Vigdergauz and co-workers[30,31]. The indicated values can be obtained as particular cases of eqn. 3 (Table 1).

TABLE 1

RELATIVE RETENTION VALUES

Retention value	*Parameters of eqn. 3*					*Equation*
	P	k	r_i	r_m	r_n	
Relative retention volume	1	0	V_N	0	V_{Nst}	$V_z = \frac{V_N}{V_{Nst}}$
Retention factor (Kováts' index)	100	z	$\log V_N$	$\log V_{Nz}$	$\log V_{N(z+1)}$	$I = 100z + \frac{\log V_N - \log V_{Nz}}{\log V_{N(z+1)} - \log V_{Nz}}$
Arithmetical factor	100	z	V_N	V_{Nz}	$\log V_{N(z+1)}$	$A = 100z + \frac{V_N - V_{Nz}}{V_{N(z+1)} - V_{Nz}}$
Relative factor*	1	$100z$	V_N	0	V_{Nz}	$RI = 100z + \frac{V_N}{V_{Nz}}$
Relative retention index*	100	1	V_N	V_{NA}	V_{NB}	$P_{AB} = 100 + 100 \cdot \frac{V_N - V_{NA}}{V_{NB} - V_{NA}}$

* Proposed by V. G. Berezkin and J. J. Walraven.

As mainly the relative retention volume and the retention factor are used in practical chromatography, let us consider the effect of adsorption specifically for these two relative quantities. Fig. 2 shows the dependence of the retention factor for a number of compounds on squalane as a function of the retention factor of the same compounds on polyethylene glycol 400 with the use of different solid supports (in plotting the graph, use was made of experimental results obtained by Evans and

Smith[32]. It follows from Fig. 2 that the effect of the solid support on the retention factor is considerable. The change in the retention factor exceeds 100 for some compounds, depending on the solid support.

It was generally believed in chromatography until recently that the relative retention value is determined solely by the ratio of the distribution constants of the given and the standard compounds between the gas and liquid phases and hence it is the chromatographic constant of a chemical compound. Therefore, the values of the relative retention volume were used as a basis for identification of chromatographic zones, *i.e.*, qualitative chromatographic analysis of the mixtures to be separated.

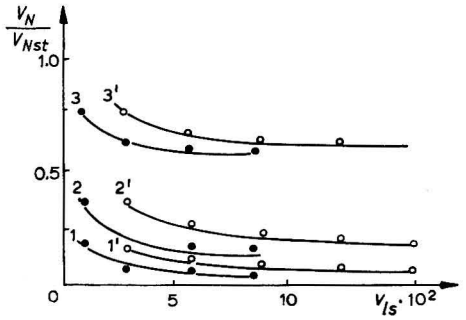

Fig. 3. Dependence of relative retention volume (standard = methyl ethyl ketone) on LSP volume (per gram of solid support) (according to data of Pecsok *et al.*[33]). Experimental conditions: LSP = β,β'-thiodipropionitrile, 25°; solid support = Chromosorb W (1–3) and refractory (1′–3′). 1,1′ = *n*-heptane; 2,2′ = *n*-butyl ethyl ether; 3,3′ = ethyl acetate.

Because of adsorption phenomena in partition chromatography, the problem of identification of chromatographic zones should be considered from another viewpoint. As a result of adsorption during chromatographic separations, the relative retention value is determined, in general, not only by the ratio of the distribution constants of the given and the standard compounds (see, for instance, ref. 1), but also by the adsorption properties of the solid support, the content of the liquid stationary phase on the solid support, the phase characteristics of the sorbent, which depend, in particular, on the conditions of its preparation, etc.[7,15]. As an example, Fig. 3 depicts the dependence of the relative retention volume on the content of the LSP on the solid support calculated by us from the data of Pecsok *et al.*[33]. It follows from the data listed that the relative retention value during adsorption in the chromatographic process is not a chromatographic constant of a compound. Indeed, in general, on the strength of eqn. 2, the relative retention can be expressed by the equation

$$\frac{V_N}{V_{Nst}} = \frac{K_l}{K_{lst}} \cdot \frac{1 + \left(\sum_{i=2}^{m} K_{li}\, v_{li} + \sum_{j=1}^{n} K_{sj}\, S_{sj}\right) \cdot \frac{1}{K_l\, v_l}}{1 + \left(\sum_{i=2}^{m} K_{list}\, v_{li} + \sum_{j=1}^{n} K_{sjst}\, S_{sj}\right) \cdot \frac{1}{K_{lst}\, v_l}} \tag{4}$$

where V_N is the net retention volume of the substance on a polyphase sorbent, V_{Nst}

is the net retention volume of the substance adopted as the standard, K_l is the equilibrium constant of the substance between the gaseous and stationary liquid phases, K_{lst} is the equilibrium constant of the standard substance between the gas phase and the LSP, K_{li} is the equilibrium constant between the mobile phase and the i-type LSP, v_{li} is the volume of the i-type LSP in the chromatographic column, K_{sj} is the equilibrium constant between the LSP and the j-type interface, and S_{sj} is the area of the j-type surface in the column. Thus, the relative retention value in the general case cannot be used for the identification of compounds on the basis of data published in the literature, as the production of sorbents having identical phase characteristics, with the same LSP distribution on the solid support in different laboratories using different batches of commercial materials, is virtually impossible in most instances.

It should be noted that the known methods for the modification of solid diatomaceous supports (for instance, silanization) or the use of polymer (PTFE) supports decrease the role of adsorption phenomena, but the contribution of adsorption to the relative retention value often remains considerable (see, for instance, refs. 34 and 35). The use of capillary columns does not solve the problem of interlaboratory reproducibility, as the contribution of adsorption still remains appreciable[36]. Therefore, for chromatography to be used extensively as a method of qualitative analysis, it is necessary to devise methods for determining the distribution constant ratio on the basis of experimentally determined relative retention values. Some of the versions of these methods, based on the use of the dependences of the relative retention values on the reciprocal of the LSP content on a solid support, are considered below.

If the retention volume is determined exclusively by the dissolution of the chromatographed substance in the LSP and the adsorption at interfaces with a mobile phase and a solid support (this is rather often the case[7,11,14,15]), eqn. 4 simplifies, and the relative retention volume can be expressed by the equation

$$\frac{V_N}{V_{Nst}} = \frac{K_l}{K_{lst}} \cdot \frac{1 + (K_{gl} S_{ls} + K_l K_s S_s)/K_l v_{ls}}{1 + (K_{glst} S_{ls} + K_{lst} K_{sst} S_s)/K_{lst} v_{ls}} \tag{5}$$

Expanding this equation in a Maclaurin series with respect to the variable $1/v_{ls}$, we can write

$$\frac{V_N}{V_{Nst}} = \frac{K_l}{K_{lst}} + \lambda \cdot \frac{1}{v_{ls}} = V^0 + \lambda \cdot \frac{1}{v_{ls}} \tag{6}$$

where

$$\lambda = \frac{(K_{gl} K_{lst} - K_{glst} K_l) S_l + (K_s - K_{sst}) K_l K_{lst} S_{ss}}{K_{lst}^2} \tag{7}$$

Note that a similar relationship is obtained when considering a more general equation (eqn. 4) if an increased LSP content on a solid support increases only the thickness of the LSP film. In Fig. 4, the experimental data of Pecsok *et al.*[33] are presented in accordance with eqn. 6. From this, it follows that the use of eqn. 6 enables the distribution constant ratio, which is independent of the experimental con-

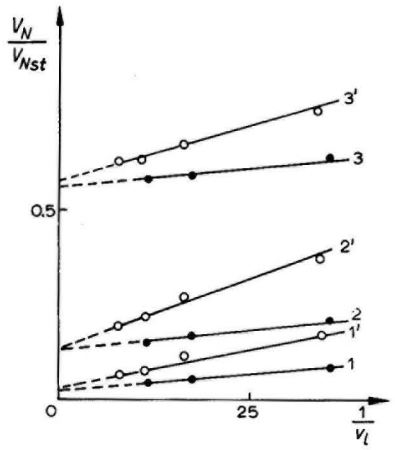

Fig. 4. Dependence of relative retention volume (standard = methyl ethyl ketone) on reciprocal of LSP volume in terms of 1 g of solid support (according to data of Pecsok *et al.*[33]). Experimental conditions and notation as in Fig. 3.

ditions, to be determined. It is advisable to choose for the standard a substance for which the retention is determined only by dissolution. In this case

$$V_{Nst} = K_{lst}\, v_l \tag{8}$$

$$\lambda_1 = \frac{K_{gl}\, S_l + K_l\, K_s\, S_s}{K_{lst}} \tag{9}$$

$$\frac{1}{v_l} = K_{lst} \cdot \frac{1}{V_{Nst}} = \frac{100\, d_l}{P_s} \cdot \frac{1}{P_l} \tag{10}$$

where v_l is the volume of the LSP in the column, d_l is the LSP density, and P_l is the percentage LSP content in the column (weight of the solid support = 100%).

Using eqns. 8–10, we can represent eqn. 6 by

$$\frac{V_N}{V_{Nst}} = \frac{K_l}{K_{lst}} + \lambda_2 \frac{1}{V_{Nst}} = \frac{K_l}{K_{lst}} + \lambda_3 \cdot \frac{1}{P_l} = V^0 + \lambda_3 \cdot \frac{1}{P_l} \tag{11}$$

Eqn. 11 can be used in those cases where the determination of v_{ls} is difficult.

In Figs. 4–6 are shown the dependences of the relative retention volume on the reciprocal of the LSP content on the solid support or on a value proportional to it (see eqn. 11, derived on the basis of the data of refs. 33 and 35). It follows from Figs. 4–6 that the proposed methods can be applied successfully to determine the distribution constant ratio, the thermodynamic characteristic of a substance from which one can identify it. The values of K_l/K_{lst} obtained are independent of the LSP content and the type of solid support.

In gas chromatography, together with the relative retention values, extensive use is made of Kováts' index system[37]:

$$I = 100z + 100 \log \left(\frac{V_N}{V_{Nz}}\right) \Big/ \log \left(\frac{V_{N(z+1)}}{V_{Nz}}\right) \tag{12}$$

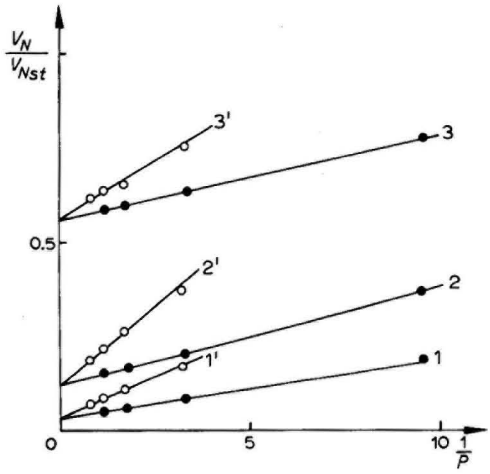

Fig. 5. Dependence of relative retention volume (standard = methyl ethyl ketone) on reciprocal of LSP content, % (according to data of Pecsok *et al.*[33]). Experimental conditions and notation as in Fig. 3.

where V_{Nz} is the net (or corrected) retention volume of an *n*-alkane whose molecule contains z carbon atoms, $V_{N\,(z+1)}$ is the net (or corrected) retention volume of an *n*-alkane whose molecule contains $z + 1$ carbon atoms, V_N is the net (or corrected) retention volume of the compound to be analyzed, and

$$V_{Nz} \leqslant V_N < V_{N\,(z+1)}$$

For the set of standard substances, it is advisable to select compounds for which the retention is determined exclusively by dissolution in the LSP. In this case,

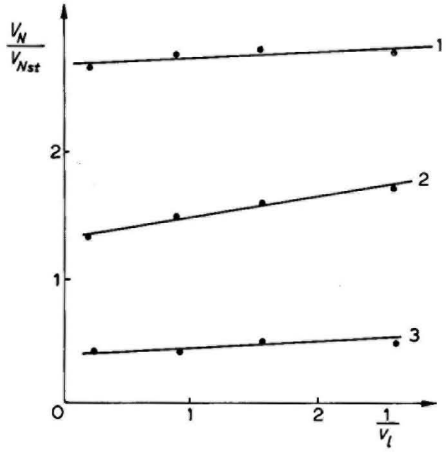

Fig. 6. Dependence of relative retention volume (standard = ethanol) on reciprocal of LSP volume (in terms of 1 g of solid support) (according to data of ref. 35). 1 = *n*-Butylamine; 2 = *n*-hexane; 3 = water. Experimental conditions: LSP = dinonyl phthalate, 86°, solid support = Teflon.

expanding the numerator of the second term of eqn. 12 in a Maclaurin series with respect to $1/v_{ls}$ and taking only the first two terms, we obtain

$$\log \frac{V_N}{V_{Nz}} = \log \left(\frac{K_l}{K_{lz}} + \lambda \cdot \frac{1}{v_{ls}} \right) = \log \left(\frac{K_l}{K_{lz}} \right) + 0.43\, \lambda_4 \cdot \frac{1}{v_{ls}} \tag{13}$$

where $\lambda_4 = \lambda \cdot K_{lz}/K_l$. Taking into account eqn. 13, we can transform eqn. 12 to give

$$I = 100z + 100 \log \left(\frac{K_l}{K_{lz}} \right) \Big/ \log \left(\frac{K_{l(z+1)}}{K_{lz}} \right) + \frac{43\, \lambda_4}{\log \left(\frac{K_{l(z+1)}}{K_{lz}} \right)} \cdot \frac{1}{v_{lz}} \tag{14}$$

or

$$I = I^0 + \lambda_4 \cdot \frac{1}{v_{ls}} \tag{15}$$

where

$$I^0 = 100z + 100 \log \left(\frac{K_l}{K_{lz}} \right) \Big/ \log \left(\frac{K_{l(z+1)}}{K_{lz}} \right) \tag{16}$$

and

$$\lambda_4 = 43\, \lambda\, K_{lz}/K_l \log \left(\frac{K_{l(z+1)}}{K_{lz}} \right) \tag{17}$$

For non-polar phases and medium-polarity phases, the requirements for standard compounds are usually met by *n*-alkanes, but in the case of polar LSP it is advisable to choose polar compounds (for instance, *n*-alcohols) as standards. As an example, Fig. 7 shows the dependence of the Kováts' indices on the reciprocal of the LSP content; in this case, the standards used were *n*-alkanes, and in another case *n*-alcohols (re-calculated by us from the data of ref. 38). From the above information, it follows that the use as standards of *n*-alcohols, whose retention, in contrast to *n*-alkanes, is largely determined by dissolution in the LSP, enabled stable Kováts' indices to be obtained for oxygen-containing compounds, but not for *n*-alkanes.

In Fig. 8, the corresponding graphs for the determination of I^0 are given for *n*-alkanes. The use of eqn. 15 enables the constant value I^0 to be determined in this case also. Similar results have been obtained upon treatment of the experimental data[39].

As an example, Fig. 9 shows the determination of the invariant value with respect to experiment for the methyl ether of myristic acid and *n*-decanol. It follows from Fig. 9 that the utilization as a solid support of a sufficiently inert Chromosorb, washed with an acid and treated with dimethyldichlorosilane, guarantees the absence of adsorption processes in GLC. Adsorption phenomena also manifest themselves in capillary chromatography[36]. In this case, however, owing to the fact that the determination of the values of v_l (or P) involves some difficulties, it is advisable to select, for values proportional to the amount of the LSP in the capillary column, the value

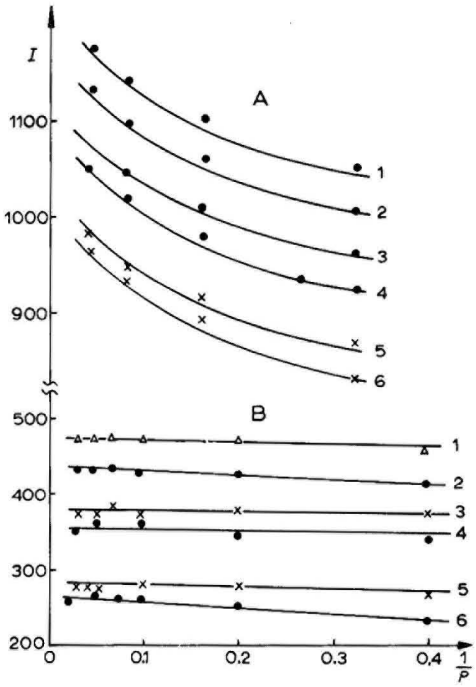

Fig. 7. Effect of choice of standard substances on dependence of retention factors upon reciprocal of LSP content (%) on solid support (according to data of refs. 38 and 39). 1 = 2-Heptanone; 2 = ethylbenzene; 3 = 2-hexanone; 4 = toluene; 5 = 2-pentanone; 6 = benzene. Standard substances: C_8–C_{12} *n*-alkanes (A) and C_2–C_4 *n*-alcohols (B). Experimental conditions: LSP = Carbowax 20M, 120°; support = Chromosorb P.

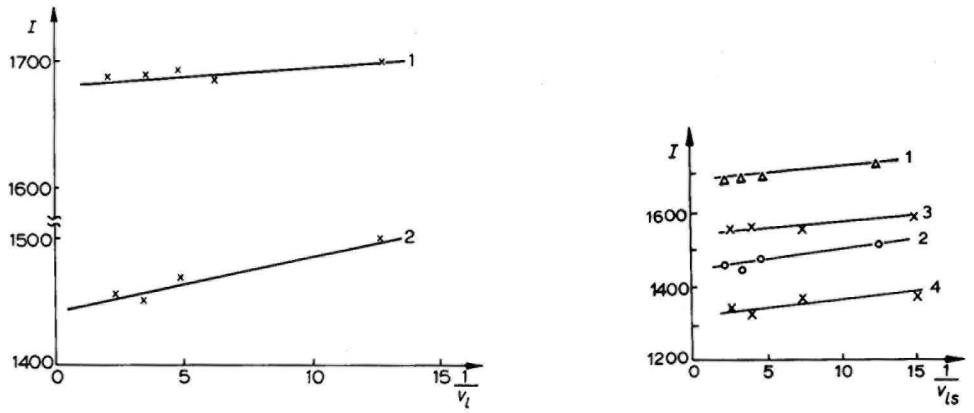

Fig. 8. Dependence of retention factors of *n*-dodecane (1) and *n*-nonane (2) (standards = C_2–C_4 *n*-alcohols) on reciprocal of LSP content on solid support (%) (according to data of refs. 38 and 39). Experimental conditions as in Fig. 7.

Fig. 9. Dependence of retention factors on reciprocal of LSP volume in column. 1 = Methyl ether of myristic acid; 2 = *n*-dodecanol, 3 = C_{12} alcohols; 4 = C_{10} alcohols. Experimental conditions: LSP for 1 and 2 = Apiezon L; LSP for 3 and 4 = silicone oil DC-550; 150°; solid support = Chromosorb G washed with acid and modified with dimethyldichlorosilane; column, 100 × 0.3 cm.

of the extraction coefficient of a standard substance whose adsorption can be neglected. In this case, we obtain eqn. 18 for the relative column volume:

$$V_z = \frac{K_{li}}{K_{lst}} + \frac{K_{gli}\,S_l + K_{li}\,K_{si}\,S_s}{V_m} \cdot \frac{1}{k_{st}} = \frac{K_{li}}{K_{lst}} + \lambda_i \cdot \frac{1}{k_{st}} = V^0 + \lambda_1 \cdot \frac{1}{k_{st}} \quad (18)$$

where

$$\lambda_i = \frac{K_{gli}\,S_l + K_{li}\,K_{si}\,S_s}{V_m} \approx \text{constant}$$

V_m is the dead volume of the column and k_{st} the capacity ratio of the standard compound.

TABLE 2

COMPARISON OF RELATIVE RETENTION VALUES CALCULATED VIA EQNS. 11 AND 20 FROM DATA IN REF. 33

Compound	*Eqn. 11*	*Eqn. 20*		*Experimental data*[33]	
		Firebrick	*Chromosorb*	*Firebrick, 8.99% LSP*	*Chromosorb, 8.75% LSP*
n-Heptane	0.015	0.016	0.019	0.079	0.045
n-Butyl ethyl ether	0.105	0.112	0.113	0.129	0.151
Ethyl acetate	0.550	0.549	0.553	0.629	0.590

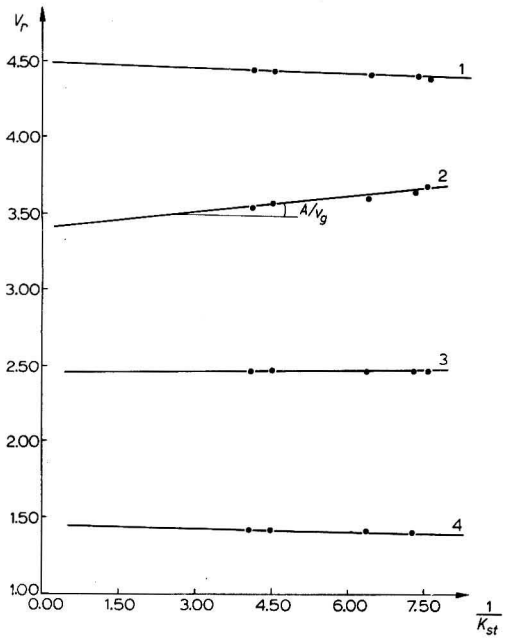

Fig. 10. Dependence of relative retention volume on reciprocal of extraction factor of capacity ratio. 1 = Cyclohexane; 2 = benzene; 3 = *n*-hexane; 4 = 2,2-dimethylbutane.

Fig. 10 shows that the relative retention volume is a linear function of the reciprocal of the capacity ratio. The intercept on the y-axis is equal to V^0.

Thus, a linear equation of the type represented by eqn. 18 can be used in capillary chromatography also.

The question naturally arises whether it is possible to reduce the experimental time by rapid measurement of I^0 and V^0. These values can be obtained by measuring I and V_r with two different contents of the LSP on the solid support:

$$I^0 = \frac{I_1 P_1 - I_2 P_2}{P_1 - P_2} \tag{19}$$

$$V^0 = \frac{V_1 P_1 - V_2 P_2}{P_1 - P_2} \tag{20}$$

where I_1 and I_2 are retention factors with LSP contents P_1 and P_2, respectively, and V_1 and V_2 are the relative retention volumes with LSP contents P_1 and P_2, respectively.

Table 2 gives the values of V^0 calculated according to data of Pecsok *et al.*[33] by eqns. 11 and 20. It follows from the data listed that both equations lead to similar results.

CONCLUSION

Accurate and meaningful relative retention data in GLC can be obtained only by correcting retention volumes for adsorption effects. In general, it is advisable to use at least two sorbents impregnated with different amounts of stationary phase to check the magnitude of the adsorption.

When the variation of the relative retention data depends appreciably upon the loading of the support, it is necessary to use one of the methods described to take adsorption into account.

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SUMMARY

Adsorption phenomena in GLC lead to a considerable inter-laboratory non-reproducibility of relative retention values on the basis of which chromatographic zones are identified. This paper considers the application, for identification purposes, of limiting relative retention values that are determined exclusively from the ratio of the distribution coefficients of the substance under study and the standard between the gas and liquid phases, and suggests methods for determining their values.

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CHREV. 79

GAS CHROMATOGRAPHIC MEASUREMENT OF TRANSPORT PROPERTIES*

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1. INTRODUCTION

The greatest emphasis during the past 15 years in the application of gas chromatography to physical-analytical measurements has been placed on the determination of activity coefficients, vapour pressures, thermodynamic quantities, boiling points, second virial coefficients of gas mixtures, kinetic constants, solid surface properties, inter- and intra-phase mass transfer coefficients, etc.[1–12]. Choudhary and Doraiswamy[12] presented a critical review of the applications of gas chromatography in catalysis.

In this paper, a comprehensive review of the literature published during the past 15 years on the use of gas chromatography in the evaluation of binary diffusion coefficients of gases and organic vapours, effective diffusivities of catalysts and adsorbents, intra-particle and intra-crystalline mass transfer coefficients, surface diffusion coefficients and heat transfer coefficients, is presented.

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2. BINARY DIFFUSION COEFFICIENTS OF GASES

Numerous techniques have been described for the measurement of binary diffusion coefficients of gases and organic vapours. Most of these methods, however, are time consuming in the evaluation of quantitative results[13]. Recent developments in the theory of chromatography[14–18] have made it possible to develop techniques[13,19] for the rapid and accurate measurement of diffusion coefficients.

A. Method of Giddings and Seager

Giddings and Seager[13,20–22] were among the first workers to apply gas chromatography in order to measure gas-phase diffusion coefficients. The method is based on the special case[15] of the more general theory of chromatography first derived by Taylor[23,24].

According to generalized chromatographic theory[15,25,26], the height equivalent to a theoretical plate (HETP, H) of a packed column can be written as

$$H = 1/[(1/2\,\lambda\, d_p) + (1/C_g\, v)] + \frac{2\,\gamma\, D_g}{v} + C_l\, v + C_k\, v \tag{1}$$

This equation is simplified by replacing the packed column by an empty tube of uniform circular cross-section and assuming that adsorption at the wall is negligible, so that $C_k = 0$. As there is no liquid in the tube, $C_l = 0$. Further, the quantity $2\,\lambda\, d_p$ approaches infinity because there are no mixing stages in the tube, and for this precise geometry γ is unity. Thus, eqn. 1, in the case of an empty tube of uniform cross-section, reduces to

$$H = \frac{r_0^2\, v}{24\, D_g} + \frac{2\, D_g}{v} \tag{2}$$

where the expression for a circular tube, $r_0^2/24\, D_g$, replaces C_g.

An equivalent form of eqn. 2, as derived by Taylor[23], can be written as

$$D_g = \frac{v}{4}\left(H \pm \sqrt{H^2 - \frac{r_0^2}{3}}\right) \tag{3}$$

For low flow-rates (v), eqn. 3 can be simplified[27] to

$$D_g = v\left(\frac{H}{2}\right) \tag{4}$$

which would be valid for low-molecular-weight gases.

The HETP can be obtained from chromatographic data by the relationship

$$H = \frac{L}{n} = \frac{L}{16}\left(\frac{\omega}{t_R}\right)^2 = \frac{L}{16}\left(\frac{m}{l}\right)^2 \tag{5}$$

or

$$H = L\left(\frac{\tau}{t_R}\right)^2 \tag{6}$$

Eqns. 3 and 5 (or 6) imply that only three quantities, the peak width (or peak variance), the retention time and the average carrier flow velocity, are required for estimating the gas phase diffusivity. The positive root of eqn. 3 is valid up to a certain critical velocity v_c given by[13]:

$$v_c = 4\sqrt{3}\, D_{AB}/r_0 \tag{7}$$

At velocities greater than v_c, the negative root becomes valid. For the case when $v \gg v_c$, the Taylor limit becomes valid.

The apparatus consists of a commercially available gas chromatographic unit, with an empty tube of uniform circular cross-section replacing the packed column. In order to correct for the end-effects and for diffusion occurring in the instrument dead volume, Giddings and Seager[13] collected data using both the long tube and the short tube separately, and the data for the short tube are subtracted from those for the long tube. Thus

$$H = (L_l - L_s)\,(\tau_l^2 - \tau_s^2)/(t_l - t_s)^2 \tag{8}$$

The need for this cumbersome two-column procedure was eliminated by Fuller *et al.*[28] through direct on-column sample introduction and a rigorous reduction of the detector dead volume. The oven dimensions were increased so as to accommodate the diffusion tube without tight coils and bends, which tend to distort tube cross-sections and otherwise alter the peak dispersion. Their gas chromatographic broadening apparatus for diffusion measurements is illustrated in Fig. 1.

Arnikar and co-workers[29,30] made use of an electrodeless discharge tube as a detector in the rapid determination of binary diffusion coefficients of some organic vapours into nitrogen.

Hargrave and Sawyer[31] applied a similar gas chromatographic method for the determination of gaseous inter-diffusion coefficients for solute vapour–carrier gas pairs in the temperature range 25–250° by using eqn. 4. The plate height is calculated from the relationship

$$H = \frac{L}{5.545} \cdot \left[\frac{\omega}{t_R}\right] \tag{9}$$

and the flow velocity (v) at the column outlet is calculated from

$$v = \frac{L}{t_R} \tag{10}$$

as there is no measurable pressure drop across an open column.

Fuller *et al.*[28] determined experimental binary diffusion coefficients for some halogenated hydrocarbon compounds diffusing into helium by utilizing the gas chromatographic peak broadening technique, and described certain modifications that have improved the speed and accuracy of the technique. A critical discussion of the validity of the method is also given.

Huang *et al.*[32] obtained experimental diffusivities for several gas–gas, gas–

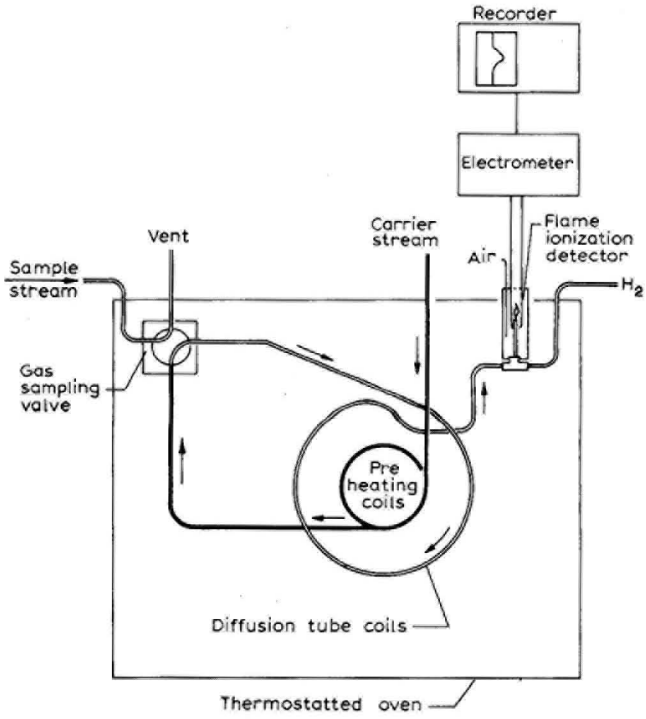

Fig. 1. GC broadening apparatus for diffusion measurements[28].

liquid vapour and gas–solid vapour systems, using the above method. On the basis of the experimental results, a generalised semi-empirical equation:

$$D_{AB} = \frac{5.06\, T^{1.75}}{(V_A^{1/3} + V_B^{1/3})^2\, p^{1.286}} \left(\frac{1}{M_A} + \frac{1}{M_B}\right)^{1/2} \tag{11}$$

has been developed to predict the diffusivities of binary gas–gas and gas–liquid vapour systems as a function of pressure and temperature. A comparison of this equation with data appearing in the literature yields an average error of 3.5%, which is less than that predicted by many other methods.

Kwok *et al.*[33] suggested that the normal plate height expression can still be used if proper modification of the diffusion coefficient is made in the case of linear adsorption. Several investigators[27,31,34–38] have established the validity of the above gas chromatographic open-tube method for the measurement of the inter-diffusion coefficients of gases and vapours of volatile organic compounds.

B. Method of Carberry and Bretton

Carberry and Bretton[39] suggested a pulse-flow method, according to which the inter-diffusion coefficient can be obtained from the concentration–time distribution data or from the mean retention time as measured at the diffuse pulse maxima:

$$t_m = \frac{D_g}{v^2}\left[\left(1 + \frac{L\,v}{D_g}\right)^{1/2} - 1\right] \tag{12}$$

For beds of more than several particle diameters in length, eqn. 12 becomes a simple function of D_g, tube length (L) and velocity (v):

$$t_m = \frac{L}{v} - \frac{D_g}{v^2} = \theta - \frac{D_g}{v^2} \tag{13}$$

or

$$\frac{t_m}{\theta} = -\frac{D_g}{L\,v} \tag{14}$$

Thus a plot of the ratio of the pulse retention time to that of carrier velocity, t_m/θ, is a function of $1/L\,v$, the slope being equal to the diffusion coefficient. Beyond the flow regime where molecular transport governs axial dispersion, D_g is directly proportional to $L\,v$, and t_m/θ therefore becomes constant with the onset of turbulent diffusion.

Frontal analysis was also used by Fejes and Schay[40] for the determination of gaseous inter-diffusion coefficients.

All the methods discussed above are applicable to both open and packed tubes, but suffer from the disadvantages inherent in operating at low flow-rates from the uncertainty as to the role of "eddy diffusion", which may interact in a complex way with gas-phase mass transfer[41].

C. Arrested elution method

Knox and McLaren[42] suggested an arrested elution method that by-passes most of the experimental and theoretical difficulties which occur in the continuous elution method of Giddings and Seager[13] and Giddings[14–18]. It is applicable equally to open and packed columns and is of intrinsically higher precision than the continuous method based upon HETP measurement.

In this method, a sharp band of an unsorbed gas is injected into the column and eluted at a controlled and measurable velocity. When the band is about half way down the column, the gas flow is arrested for a time t, during which spreading can occur only by diffusion. Finally, the band is eluted from the column and its profile and standard deviation are determined by the detector. The diffusion spreading for empty and packed tubes is given by

$$\frac{\mathrm{d}\,\delta_t^2}{\mathrm{d}\,t} = 2\,D_g/v^2 \text{ (for an empty tube)} \tag{15}$$

and

$$\frac{\mathrm{d}\,\delta_t^2}{\mathrm{d}\,t} = 2\,\gamma\,D_g/v^2 \text{ (for a packed tube)} \tag{16}$$

Hence, a plot of δ_t^2 against the time of residence in the column is a straight line of slope $2\,D_g/v^2$ (for an empty tube) or $2\,\gamma\,D_g/v^2$ (for a packed column), from which the inter-diffusion coefficient can be calculated. Details of the method and the theoretical interpretation of γ are discussed in the original paper[42].

D. Multi-flow-rate and one-flow-rate methods

Huber and van Vught[36] and Kobayashi and co-workers[43–45] studied the gaseous diffusion coefficients of dilute and moderately dense gases by perturbation chromatography. Their measurements were based on the dispersion model for laminar flow in a long circular tube at up to 60 atm at 25° for tritiated methane in methane and in tetrafluoromethane by the multi-flow-rate method using the relationship[45]

$$\frac{\Delta[\delta^2]}{\Delta t} = \frac{2}{v^2} \cdot D_g + \frac{\beta}{D_g} \tag{17}$$

The diffusion of argon, methane, nitrogen and carbon dioxide in helium at 50°, 25°, 0° and −25°, respectively, at up to 60 atm was measured by the one-flow-rate method using the relationship[45]

$$\frac{\Delta\delta^2}{\Delta t} = \frac{2}{v^2} \cdot D_g + \frac{r_0}{24\, D_g} \tag{18}$$

A representative plot of the rate of change of variance $\Delta\delta^2/\Delta t$ *versus* $2/v^2$ is given in Fig. 2.

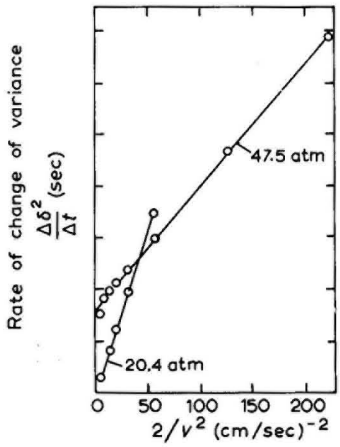

Fig. 2. Determination of diffusivity of tritiated CH_4 in CF_4 at 25° (ref. 45). $\Delta\delta^2/\Delta t = (2/v^2)D_g + \beta/D_g$.

Hu[44] and Hu and Kobayashi[45] improved perturbation detection by using a small-volume ionization chamber for radioactive detection and a micro-scale thermal conductivity cell for non-radioactive detection. Agreement between their experimental and theoretical values was good for variance calculations. Some results were obtained by using both the multi-flow-rate method, which used several velocities at the same temperature and pressure, and the one-flow-rate method, which required measurement at only one flow-rate.

Apart from the literature discussed above, several investigations have been reported on the use of gas chromatographic methods for measuring gas-phase diffusion coefficients using packed or unpacked columns[46–49].

3. EFFECTIVE DIFFUSIVITY OF CATALYSTS

The measurement of effective diffusion coefficients is based on the well known equation developed by Van Deemter *et al.*[50] for a gas chromatographic column:

$$H = A + B/v + C\,v \tag{19}$$

where H is the HETP and v is the interstitial carrier gas velocity. The terms A, B and C are constants of the column, gases and operating conditions. The term A is due to turbulence in the gas stream caused by the particles of packing and is the so-called "eddy diffusion" term. The term B is due to longitudinal diffusion of gas in the pulse both forwards and backwards in the carrier gas stream. The C term is due to mass transfer between the gas and solid phases. It can be seen from eqn. 19 that at high gas velocities the term C is dominant and the B term becomes unimportant. Thus, the equation for H at high carrier gas velocities reduces to

$$H = A + C\,v \tag{20}$$

Habgood and Hanlan[51] obtained the following expression for C:

$$C = \frac{d_p^2}{\sqrt{2}\,\pi^2} \cdot \frac{F_1}{F_2} \cdot \frac{1}{p_v\,D_e} \cdot \frac{1}{[1 + 273\,F_1/\alpha'\,T\,\varrho_b]^2} \tag{21}$$

where

$$\alpha' = \frac{[V_R^0 - V_d^0]}{W} \cdot \frac{273}{T} \tag{22}$$

They calculated the HETP for very small sample volumes giving symmetrical Gaussian peaks by the relationship

$$H = \frac{L}{16}\left(\frac{\Delta S^0}{\Delta V_R^0}\right)^2 \tag{23}$$

and attempted a tentative interpretation of C in terms of an apparent gas phase diffusion in the charcoal pores.

The term C can also be expressed[52] as

$$C = \frac{d_p^2}{\sqrt{2}\,\pi^2} \cdot \frac{F_1}{F_2} \cdot \frac{1}{p_v\,D_e} \cdot \frac{1}{[1 + K_0\,F_1/F_2]^2} \tag{24}$$

where

$$K_0 = 1/[\varepsilon + (t_m - t_d)\,v\,F_1/F_2\,L] \tag{25}$$

and the HETP calculated by measuring the pulse broadening of a non-chemically interacting gas at various flow velocities that occur in a column packed with catalyst using eqn. 5, 6 or 23.

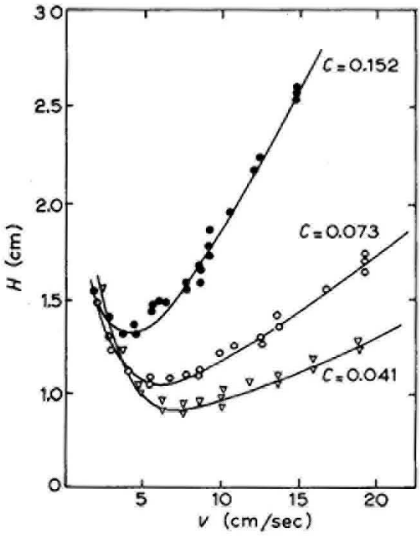

Fig. 3. HETP as a function of interstitial gas velocity for SO_2 on Na-mordenite[53]. Temperature: ●, 133°; ○, 210°; ▽, 261°.

Using the values of H as a function of flow velocity, the term C (and also A and B) of the Van Deemter equation can be calculated by plotting the HETP, H, against the flow velocity (or by the least-squares method); the slope of the linear portion of the curve in the region of high gas velocities corresponds to the value of the term C (a representative plot of H *versus* v is shown in Fig. 3[53]). Hence, as all the terms in eqn. 24 except D_e can be determined experimentally, the effective diffusivity of the catalyst can be calculated with sufficient accuracy.

Leffler[52] applied this method to alumina H-51 using nitrogen as the adsorbed gas at −78° and found reasonable agreement between the results obtained from this method and the Weisz and Schwartz relationship[54]. Davis and Scott[55], using gas chromatography, also reported good agreement between the values of D_e and steady-state results for solids that are reasonably homogeneous.

Szlaur[56] measured the adsorption kinetics of polar compounds on molecular sieves in order to determine the effective diffusion coefficients into granules. Values of the coefficients of mass transfer for packed capillary gas–solid chromatographic columns were measured from the dependence of HETP on flow-rate and used to explain the low values of zone diffusion[57].

Eberly[58] and Ma and Mancel[53] studied the effective diffusivity of inert gases (argon, krypton and sulphur hexafluoride) in a series of Na- and H-mordenites, Na-faujasite and amorphous silica–alumina catalysts and of carbon dioxide, nitrogen dioxide and sulphur dioxide in molecular sieve zeolites. They found that this gas chromatographic pulse technique is especially suitable for measurement of effective diffusivities of solid catalysts at high temperatures and low concentrations. It is very difficult to make measurements under these conditions by the conventional vacuum technique[59]. Trimm and Corrie[58] employed this method for the measurement of the diffusivity of various gases in catalyst pellets as a function of temperature. They found

that the results obtained at low temperatures for some gases cannot be extrapolated to the higher temperatures at which the catalyst operates. At temperatures below 400° the variation of D_e with temperature showed predictable behaviour, but above 400° a marked increase in the dependence of D_e on temperature was observed, the magnitude of which depended on the nature of the gas. They explained this anomalous effect in terms of a surface diffusion model. As surface mobility is known to increase exponentially with temperature, surface diffusion provides a good explanation of their results.

This gas chromatographic method for the determination of D_e has the inherent advantage that measurements are made at temperatures which approach those used in commercial processes. Detailed procedures for the determination of the experimental values of the terms in the expression used for calculating effective diffusion coefficients have been given by Leffler[52], Eberly[58] and Ma and Mancel[53].

4. MASS TRANSFER COEFFICIENTS FOR PACKED BEDS

A. General

The advanced theory of gas chromatography permits the estimation of the mass transfer resistances such as axial diffusion, interphase diffusion, film diffusion and intra-particle (or intra-crystalline) diffusion, by chromatographic measurements[60–64]. Recent developments in gas chromatographic theory, with emphasis on the mass transfer processes that occur in the packed column, are summarized below.

Following the simple approach of Giddings[65] and Van Deemter *et al.*[50], Jones[25] derived a generalized expression for the HETP including the effect of gas-phase mass transfer. Van Deemter's original equation (eqn. 19) was modified by Kambara and co-workers[66,67], who gave a mathematical treatment of the kinetic role of diffusion and the pressure drop in gas chromatography. There are a number of papers and theories suggesting different modifications of Van Deemter's equation[68–70]. Takács[70] developed an approximation equation which involves the use of a simple mathematical method for numerically determining the coefficients of the approximation equation by means of a computer.

Giddings[15,71] carried out theoretical studies of band spreading in chromatography by a non-equilibrium method[14,16] and examined the contribution of lateral diffusion to plate height. Khan[72] presented a non-equilibrium treatment which includes interfacial mass transfer resistance. Kubin[73] contributed to the theory of chromatography in a detailed analysis of diffusion outside and inside the support particles.

At low concentrations, the shapes of the peaks deviate from a Gaussian distribution[74] and may be sensitive to the detailed kinetics. Bock and Parke[75] calculated the first four moments of the distribution function on the assumption that the rates of sorption and desorption do not play significant roles, and the distribution functions were found from these moments. Chromatographic peaks were treated mathematically by Kaminskii *et al.*[76] and the first six moments of the peak were derived on the assumption that peak spreading is determined solely by diffusion in the stationary phase and the sorption coefficient is large; a method for the recalculation of the distribution function from the moment was also suggested. Linear non-

equilibrium chromatography was treated mathematically independently by Kučera[77], and five moments of the chromatographic peak were calculated and their physical significance was discussed. Grubner[78] applied the statistical moments theory to the solution of a system of partial differential equations describing a model based on diffusion-controlled kinetics for gas–solid chromatography. Grubner and Underhill[79] used a standard mathematical method of analysis by moments to compare the most common equations for mass transfer in a packed bed, and the first six moments were given for four equations (for a theoretical chamber model, film-limited mass transfer, intra-particle diffusion and inter-particle diffusion). In each instance, the first ordinary moment was found to be independent of the mechanism of mass transfer.

Grubner *et al.*[80] studied mass transfer phenomena in gas–solid systems with special emphasis on the internal porosity of the stationary phase, and the mass transfer coefficients were obtained from a more exact treatment of gas–solid chromatography[81].

Analysis of moments is a very powerful mathematical technique and can be used as a means of comparing the previously derived equations for break-through curves[60,61,79]. From an experimental viewpoint, only the first five or six moments need be calculated; the higher moments are difficult to determine from experimental data[79].

B. Method based on moment analysis

Schneider and Smith[60,61] presented a new method for determining adsorption equilibrium constants, rate constants, mass transfer coefficients, axial or longitudinal diffusivity, intra-particle diffusivity and surface diffusivity from gas chromatographic data. The method was based upon the theory of chromatography developed by Kubin[73] and Kučera[77] for relating the moments of the effluent concentration wave from a bed of adsorbent particles to the rate constants associated with various steps in the overall adsorption process. Basically, a pulse of the adsorbate is injected into the column (packed with catalyst particles) head and the first and second moments of the outlet peak are measured. Theory permits the calculation of the mass transfer coefficients from these measurements. The principles of the method are as follows.

The first absolute moment (μ_1') of the chromatographic curve is defined as

$$\mu_1' = m_1/m_0 \tag{26a}$$

where

$$m_n = \int_0^\infty t^n\, c(z,t)\, \mathrm{d}t \quad (n = 0, 1, 2, \ldots) \tag{26b}$$

The first moment is of basic significance in the determination of retention time and depends only on the partition coefficient and on the longitudinal diffusion, and is not affected by transport phenomena on the grain and across the surface film or by the shape and size of the grain.

The second central moment (μ_1') of the chromatographic curve is defined as

$$\mu_2 = \frac{1}{m_0} \int_0^\infty (t - \mu_1')^2\, c(z,t)\, \mathrm{d}t \tag{27}$$

The second central moment has significance in the determination of the peak width and, as with all higher moments, it depends on all factors that characterize the transport of a given compound through the column.

The moments μ_1' and μ_2 can be evaluated explicitly, using Laplace–Carson transforms[60,61], as

$$\mu_1' = \frac{z}{v}(1 + \delta_0) + \frac{t_{0A}}{2} \tag{28}$$

and

$$\mu_2 = \frac{2z}{v}\left[\delta_1 + \frac{E_A}{\alpha}\left(1 + \delta_0\right)^2 \frac{1}{v^2}\right] + \frac{t_{0A}^2}{12} \tag{29}$$

where

$$\delta_0 = \frac{1 - \alpha}{\alpha} \cdot \beta \left(1 + \frac{\varrho_p}{\beta} \cdot K_A\right) \tag{30}$$

and

$$\delta_1 = \frac{1 - \alpha}{\alpha} \cdot \frac{R^2 \beta^2}{15} \cdot \left(1 + \frac{\varrho_p}{\beta} K_A\right)^2 \cdot \left(\frac{1}{D_p} + \frac{5}{k_f R}\right) \tag{31}$$

Further, the effective intraparticle diffusion coefficient, D_p, is related to the effective surface diffusion coefficient, D_s, by the relationship

$$D_p = D_k + \frac{\varrho_p}{\beta} \cdot K_A D_s \tag{32}$$

and the effective gas diffusion coefficient (Knudsen), D_k, is given by

$$D_k = \frac{\beta}{q_{\text{int}}} \cdot \mathscr{D}_k \tag{33}$$

where

$$\mathscr{D}_k = 4/3\, r_p \sqrt{2\, RT/\pi M} \tag{34}$$

The effective surface diffusion coefficient, D_s, is related to the true surface diffusion coefficient, $\mathscr{D}_s$, by the relationship

$$D_s = \frac{\beta}{q_{\text{sur}}} \cdot \mathscr{D}_s \tag{35}$$

It has been shown that the external mass transfer coefficient, k_f, does not depend on the carrier gas velocity at low Reynolds number[60]. Hence k_f can be calculated from the relationship $N_{\text{sh}AB} = 2.0$, where the Sherwood number is $N_{\text{sh}AB} = 2\, R\, k_f / D_{AB}$.

By using the resulting relationship $k_f R = D_{AB}$ in eqn. 31, the effective intraparticle diffusion coefficient, D_p, can be calculated from the experimental second central moment (μ_2) for a series of chromatographic curves measured for different

carrier gas velocities, v. The adsorption coefficient, K_A, can be evaluated by means of eqns. 28 and 30 from the experimental first absolute moment (μ_1') using the same series of chromatographic curves. The binary diffusion coefficient, D_{AB}, can be calculated from theoretical equations[82] if the experimental values are not available.

Further, the axial diffusion coefficient (D_A) can be obtained from the experimental second central moments measured at high carrier gas velocities (*i.e.*, high Reynolds numbers) by eqn. 29. Finally, eqns. 32–35 are used to evaluate the effective and true surface diffusivities from the effective intra-particle diffusion coefficient, D_p.

Schneider and Smith[60,61] used the above gas chromatographic method for measuring axial dispersion coefficients, intra-particle diffusivities, external mass transfer coefficients and surface diffusivities for ethane, propane and *n*-butane on silica gel at 50–200° and at atmospheric pressure. Good agreement was obtained between the experimental and calculated breakthrough curves, which confirmed the theory of gas–solid chromatography as well as the model used for the adsorber. This fact also suggested that the rate constants determined by gas chromatography have a physical significance and are not simply empirical constants.

As the average surface coverage is very low, the surface diffusion coefficients obtained by this method are very close to the limiting values. The detailed experimental procedures, evaluation of moments of the chromatographic curves and estimation of mass transport coefficients are discussed in the original papers[60,61].

C. Method based on Fourier analysis

Gangwal *et al.*[64] obtained transport rate coefficients by determining the coefficients of the Fourier series which describes chromatographic peaks rather than the moments themselves, arguing that these can be determined more accurately than the moments, particularly higher moments. The principles of this method are as follows.

A function f(t) in an interval in time, t, of period T', can be represented by a Fourier series as

$$\mathrm{f}(t) = \sum_{n=1}^{\infty} a_n \sin(n\pi\, t/T') + \sum_{n=0}^{\infty} b_n \cos(n\pi\, t/T') \tag{36}$$

where the coefficients a_n, b_n and b_0 are

$$a_n = \frac{1}{T'} \int_0^{2T'} \mathrm{f}(t) \sin(n\pi\, t/T')\, \mathrm{d}t \tag{37}$$

$$b_n = \frac{1}{T'} \int_0^{2T'} \mathrm{f}(t) \cos(n\pi\, t/T')\, \mathrm{d}t \tag{38}$$

$$b_0 = \frac{1}{2T'} \int_0^{2T'} \mathrm{f}(t)\, \mathrm{d}t \tag{39}$$

There will be just one peak for chromatographic curves of interest in rate measurement and the period T' is a time sufficiently long to allow the tail of the peak to vanish. With injection of a sample in the carrier gas, the peak measures $c(t)$. Assuming that

the injection is closely approximated by the Kronecker delta function $\delta(t = 0)$, the normalized response is $E(t)$, the probability density function of residence time. The Fourier transform of $E(t)$ is

$$\bar{E}(i\omega') = \int_0^{2T'} E(t) \cos(n\pi t/T')\, dt - i \int_0^{2T'} E(t) \sin(n\pi t/T')\, dt \tag{40}$$

$$= T' b_n - i\, T' a_n \tag{41}$$

By employing the amplitude ratio, A_n, and the phase lag, φ_n, for the nth-harmonic component in place of a_n and b_n, we obtain

$$A_n = T' \sqrt{a_n^2 + b_n^2} \tag{42}$$

$$\varphi = \arctan(b_n/a_n) \tag{43}$$

The coefficients a_n, b_n and subsequently A_n and φ_n are to be evaluated from the normalized chromatographic curve by eqns. 37, 38, 42 and 43. The partial differential equations and boundary conditions for the system (assuming a δ input) are normalized and subjected to a Fourier transform and the coefficients a_n and b_n are obtained from the imaginary and real parts of eqn. 41. Expressions for A_n and φ_n are obtained through eqns. 42 and 43. Model parameters are then evaluated by minimizing $[(A_n)_{\text{exptl.}} - (A_n)_{\text{model}}]^2$ or $[(\varphi_n)_{\text{exptl.}} - (\varphi_n)_{\text{model}}]^2$ or their sum by a suitable search technique.

Gangwal *et al.*[64] obtained the system properties from the amplitude ratio by a five-dimensional search using the modified simplex method of Nelder and Mead[83]. The parameters evaluated were relatively independent of the choice of the Fourier coefficient but the dispersion coefficient seemed to be sensitive to the choice of amplitude ratio or phase lag. The detailed theory and the procedures for the evaluation of the parameters D, D_e, k_f, K_A and k_a of gas–solid systems from A_n (or φ_n) for a chromatographic peak by a five-dimensional search were described in the original paper[64].

The advantage of Fourier analysis over moment analysis is that it permits more information to be extracted from a curve. The higher moments (third to fifth) which are subject to increasingly large errors, or additional measurements, as Schneider and Smith[60] undertook, are necessary for moment analysis to give the same result.

Moment analysis and Fourier analysis are limited to systems that can be described by linear differential equations.

D. Method based on the passage of a pulse through a stirred reactor

The above two gas chromatographic techniques[60,61,64] can be used to study any reactant or product that emerges from the column as a reasonably well defined peak. In the study of a reactant under actual catalytic (or reaction) conditions, the product peak may interfere with that of the reactant.

Kelly and Fuller[63] proposed a method based on the passage of a pulse through

a stirred reactor to overcome this difficulty by using a more specific detector. A dynamic mathematical model of an isothermal heterogeneous continuously stirred tank catalytic reactor for a first-order reaction was developed and employed for the experimental determination of intra-granular diffusivities, binary sorption isotherms and reaction rate constants under actual reaction conditions. This method is rapid and only zero- and first-moment calculations of the transient response are required for the data analysis.

E. Study of mass transfer resistances in molecular sieves

Zikanova[84] determined the radial coefficient of internal diffusion of pentane in granulated zeolites from the dependence of statistical moments of the elution curve on the linear flow velocity of the carrier gas. The results obtained provide evidence that the overall transport rate of the sorbate molecules from the surface of the granule to the adsorption site is limited by activated diffusion within zeolite crystallites. Several investigations have been reported on the measurement of effective diffusivities of synthetic zeolite and molecular sieve catalysts using gas chromatography[53,56,58].

MacDonald and Habgood[62] described a gas chromatographic method based on the theory of Giddings and Schettler[85] for the determination of intra-crystalline mass transfer resistances in zeolite catalysts. They reported experimental results for benzene, octane and decane on an NaX zeolite catalyst at about 400°. The method is based on the following considerations.

The mass transfer in a microporous solid (such as molecular sieve and zeolite catalysts) takes place by two distinct processes: (*i*) mass transfer from the moving gas stream through the stationary gas film and within the macropore system of the granule to the external surface of the crystallite, and (*ii*) mass transfer through the micropores of the crystallite to reaction sites on the walls of the micropores. These two processes of mass transport in the regular crystal structure of the zeolites can be studied by the method of Giddings and Schettler[85], which involves measurements of the total mass transfer resistances using two carrier gases of different diffusivities. The resistance due to process *i* depends upon the nature of the carrier gas, while that due to process *ii* does not, provided that the carrier gas is not significantly adsorbed. The relative contributions of film and macropore resistances to process *i* could be obtained to some extent from results with granules of different size ranges.

According to Giddings and Schettler[85], the expression for HETP is

$$H/f_1 = H_g + C_s\, v_0\, (f_2/f_1) \tag{44}$$

where H_g is the sum of all gas-phase contributions to the HETP, C_s the intra-crystalline mass transfer coefficient, v_0 the column outlet velocity, f_2 the James–Martin pressure gradient correction used in gas chromatography to allow for the variation in local gas velocity along the column, and f_1 a factor to allow for decompression along the column. The terms contained in H_g are a function of v_0/D_g. The principle of the method lies in the fact that a known change in D_g which gives a corresponding change in H_g is produced, while not affecting C_s. By introducing a variable

$$X = v\, P_0/D_g' \tag{45}$$

eqn. 44 becomes

$$H/f_1 = H_g(X) + C_s X f_2 D'_g/f_1 P_0 \tag{46}$$

Either by varying P_0 using the same carrier gas, or by choosing two carrier gases of different diffusivities, two different curves of H/f_1 *versus* X can be obtained. The HETP can be obtained by using the relationship

$$H = L\mu_2/(\mu'_1)^2 \tag{47}$$

based on the more refined theories[78] from the statistical moments of the peak, which can readily be calculated by computer from a digitized peak[62]. By determining the HETPs over a range of flow-rates in each carrier gas, under conditions such that the range of X is the same for each gas, $H_g(X)$ will have an identical value in each carrier and hence the term C_s can be obtained from the relationship

$$C_s = [(H/f_1)_1 - (H/f_1)_2]/[X(Z_1 - Z_2)] \tag{48}$$

where

$$Z = f_2 D'_g/f_1 P_0 \tag{49}$$

Hence, from the plots of H/f_1 *versus* X and Z *versus* X, one can obtain C_s from the differences in H/f_1 and Z for two carrier gases at a given value of X. Typical plots of data[62] according to eqn. 48 are shown in Fig. 4.

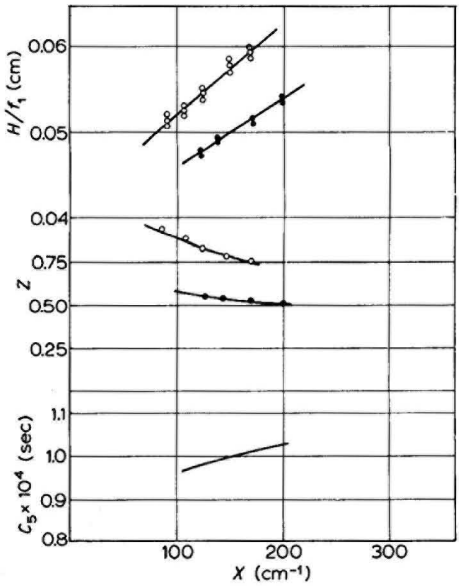

Fig. 4. Typical plots of data[62] according to eqn. 48, for benzene. Temperature = 430°. ●, Nitrogen; ○, hydrogen.

For a homogeneous spherical crystallite, an apparent diffusion coefficient, D_c, of the sorbate within the crystallite can be obtained from[15]

$$D_c = \frac{1}{30} \cdot \frac{k}{[1+k]^2} \cdot \frac{d_c^2}{C_s} \tag{50}$$

where k is the partition ratio and d_c the diameter of the zeolite crystallite. For large values of k, eqn. 50 reduces to

$$D_c = \frac{1}{30} \cdot \frac{d_c^2}{k\, C_s} \tag{51}$$

Based on their experience, MacDonald and Habgood[62] suggested criteria to indicate acceptable results.

5. HEAT TRANSFER PARAMETERS FOR PACKED BEDS

Sagara *et al.*[86] developed a temperature pulse method based on the chromatographic theory of Kubin[73] for the determination of heat transfer parameters for flow in packed beds of porous or non-porous solid particles. The method is based on the assumption that heat is dispersed axially and heat transfer occurs between fluid and particle, and intra-particle. The contribution to heat transfer due to solid-to-solid conduction, which is significant for small particle sizes, is neglected and hence this method is not suitable for beds of small particles.

The moments of the response of the column effluent temperature to a pulse in the inlet temperature are related to the thermal parameters by simple algebraic equations, so that the functional relationships of the parameters and variables are directly displaced; by varying the conditions, some of the coefficients can be determined. The first moment of the temperature curve from the bed gives reasonable estimates of the specific heat, while the second moment provides information on rate parameters (such as fluid-to-particle heat transfer coefficient, effective thermal conductivity of the particles and axial conductivity of the fluid phase). The method is rapid and interpretation of the data can easily be carried out as only elementary calculations are involved.

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7. SUMMARY

A comprehensive review on the use of gas chromatographic techniques in the measurement of binary diffusion coefficients of gases and vapours, effective diffusivities of catalysts, intra-particle and intra-crystalline mass transfer coefficients, axial diffusion coefficients, surface diffusivities and heat transfer coefficients is presented.

8. NOMENCLATURE

a_n	Fourier coefficient defined by eqn. 37.
A, B, C	constants of eqn. 19.
A_n	amplitude ratio defined by eqn. 42.
b_0, b_n	Fourier coefficients defined by eqns. 38 and 39.
c	chart paper speed of recorder.
C_g	non-equilibrium term for gaseous diffusion.
C_k	non-equilibrium term for kinetic processes.
C_l	non-equilibrium term for liquid diffusion.
C_s	mass transfer resistance within crystallite.
d_c	crystallite diameter.
d_p	particle diameter.
D	dispersion coefficient.
D_{AB}, D_g	gas-phase diffusion coefficients.
D_c	intra-crystalline diffusion coefficient.
D_e	effective diffusivity of catalyst.
D'_g	binary diffusion coefficient (at 1 atm pressure).
D_k	effective gas (Knudsen) diffusion coefficient.
$\mathscr{D}_k$	Knudsen diffusion coefficient.
D_s	effective surface diffusion coefficient.
$\mathscr{D}_s$	true surface diffusion coefficient.
E_A	apparent activation energy.
$E(i\omega')$	Fourier transform of $E(t)$.
$E(t)$	probability density residence time.
f_1, f_2	pressure correction terms.
F_1	void fraction in packing.
F_2	solid fraction in packing $(1 - F_1)$.
H	height equivalent to a theoretical plate (HETP).
H_g	plate height to gas phase contribution.
k	partition ratio.
k_a	adsorption rate constant.
k_f	external mass transfer coefficient.
K_A	adsorption coefficient.
l	distance on recorder chart measured from the point corresponding to the start of the chromatogram to the peak maximum.
L	length of packed column.
L_l	length of long diffusion tube.
L_s	length of short diffusion tube.
m	distance on the base line between points where the two tangents (to the point of inflection of peak) intersect.
M, M_A, M_B	molecular weights.
n	number of plates.
p	pressure.
p_v	pore volume.
P_0	ratio of outlet pressure to unit pressure.
q_{int}	tortuosity factor for intra-particle gas diffusion.

q_{sur}	tortuosity factor for intra-particle surface diffusion.
r_0	tube radius.
r_p	average pore radius.
R	gas constant.
ΔV_R^0	volume of carrier gas corresponding to the maximum of the peak.
ΔS^0	volume of carrier gas corresponding to the base of the elution peak.
t	time.
t_m, t_d	retention times for adsorbate and non-adsorbate.
t_{0A}	injection time for adsorbable substance.
t_R	retention time of peak measured at its centre.
T	temperature.
T'	period.
v	average carrier gas velocity.
v_c	critical velocity.
v_0	outlet velocity.
V_A, V_B	molar volumes.
V_d^0	retention volume for non-adsorbate.
V_R^0	retention volume for adsorbate.
W	weight of adsorbent.
X	reduced velocity equal to $v_0 P_0/D'_g$.
z	coordinate along the length of chromatographic column.
Z	$f_2\, D'_g/f_1\, P_0$.
α	external void fraction.
α'	distribution coefficient.
β	intra-particle void fraction.
γ, λ	constants (of the order of unity).
μ_1'	first absolute moment.
μ_2, μ_3	second and third central moments.
ϱ_b	density of packed bed.
ϱ_p	particle density.
δ^2	variance.
τ	standard deviation.
ω	peak width.
ω'	frequency (equal to $n\pi/T'$).
θ	equal to L/v.
ε	pellet porosity.

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DETERMINATION OF SECOND-INTERACTION VIRIAL COEFFICIENTS BY GAS–LIQUID CHROMATOGRAPHY

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1. INTRODUCTION

A. What are virial coefficients?

The ideal gas law, given by the familiar equation $PV = nRT$, is based on an ideal model; however, real gases generally fail to obey it. Rather than formulate a new law, however, the ideal gas law is modified so as to conform more closely to the actual behavior of gases. Several such "modified" laws have been used, including the Van der Waals, Dieterici, Berthelot, and Beattie–Bridgeman equations[1]. An alternate form is the virial expansion of the ideal gas law, which for one mole of gas becomes:

$$PV = RT\left(1 + \frac{B}{V} + \frac{C}{V^2} + \frac{D}{V^3} + \cdots\right) \tag{1}$$

where B, C, D, . . . are called the second, third, fourth, . . . virial coefficients. Obviously, given enough coefficients, the experimental data for any gas can be fitted to the ideal, albeit modified, gas law.

Eqn. 1 gives the gas law as an expansion of volume terms, so that B, C, D, . . . are a function of temperature only. The expansion could just as easily be written in terms of pressure, however:

$$PV = RT + BP + CP^2 + DP^3 + \ldots \tag{2}$$

Eqns. 1 and 2 are used interchangeably in the literature; they are easily related by setting $P = (RT/V)(1 + B/V)$ in eqn. 1 (ignoring higher coefficients) and substituting for P in the right-hand side of eqn. 2.

Virial coefficients are of more than superficial importance for chemists. For example, the true fugacity of a real gas is given by the expression[2]:

$$\ln f = \ln P + \frac{BP}{RT} + \tfrac{1}{2}(C - B^2)\left(\frac{P}{RT}\right)^2 + \cdots \tag{3}$$

Furthermore, the second, third, fourth, . . . virial coefficients can be related to binary, ternary, quaternary, . . . molecular interactions via statistical mechanics[3]. Expressions for the thermodynamic properties of real gases should also contain virial terms, for example:

$$\Delta G = RT \ln P_2/P_1 \qquad \text{(ideal)} \tag{4}$$

$$\Delta G = RT \ln P_2/P_1 + B(P_2 - P_1) + \ldots \qquad \text{(real)} \tag{5}$$

Finally, since gas chromatography (GC) involves the elution of a solute with a gas, chromatographers should be aware of the effects of non-ideal carrier gas behavior on retention parameters, which in some cases can be appreciable.

B. Gaseous mixtures

The Helmholtz free energy for one mole of an ideal gas is given by[2]:

$$A = \mu^0 - RT + RT \ln\left(\frac{RT}{P^0 V}\right) \tag{6}$$

where μ^0 is the chemical potential of the gas at a standard pressure, P^0. For n moles of a gas, eqn. 6 becomes:

$$A = n\left[\mu^0 - RT + RT \ln\left(\frac{nRT}{P^0 V}\right)\right] \tag{7}$$

When the second virial coefficient (representing gas imperfections) is included, we have:

$$A = n\left[\mu^0 - RT + RT \ln\left(\frac{nRT}{P^0 V}\right)\right] + nRT\left(\frac{nB}{V}\right) \tag{8}$$

For two perfect gases in an ideal mixture, Dalton's law of partial pressures may be written as:

$$A = \sum_i n_i\left[\mu_i^0 - RT + RT \ln\left(\frac{n_i RT}{P^0 V}\right)\right] \tag{9}$$

That is, the system is described by a simple summation of the properties of each component. For a mixture of two non-ideal gases, eqn. 8 therefore becomes:

$$\begin{aligned} A = {} & n_1\left[\mu_1^0 - RT + RT \ln\left(\frac{n_1 RT}{P^0 V}\right)\right] + \\ & n_2\left[\mu_2^0 - RT + RT \ln\left(\frac{n_2 RT}{P^0 V}\right)\right] + \\ & (n_1^2 B_{11} + 2n_1 n_2 B_{12} + n_2^2 B_{22})\frac{RT}{V} \end{aligned} \tag{10}$$

where B_{11} and B_{22} are the second virial coefficients for species 1 and 2 as if each were present exclusively, and B_{12} is the cross-coefficient (or, the mixed second virial coefficient) for the two gases. That is, B_{12} represents the non-ideal interactions between species 1 molecules and species 2 molecules. The B_{12} values are often called second-interaction virial coefficients, because they represent the non-ideal gas-phase interactions between two dissimilar species.

Eqn. 10 is now differentiated with respect to volume at constant temperature to obtain[2]:

$$PV = (n_1 + n_2)\,RT + (n_1^2 B_{11} + 2n_1 n_2 B_{12} + n_2^2 B_{22})\,\frac{RT}{V} \tag{11}$$

Eqn. 11 describes the behavior of a non-ideal gaseous mixture in terms of the ideal gas law and virial coefficients, and is applicable to GC, where a gaseous solute is eluted by a carrier, also a gas. As is most often the case, solute molecules are very different from carrier species, so that the virial corrections to the ideal gas law (given by eqn. 11) may be appreciable. This is in fact the case when carrier gases other than helium or hydrogen are used at pressures greater than 2 atm, and is a contributing factor to the non-reproducibility of GC data. Much of the work by chromatographers has been oriented toward the determination of B_{12} values, however, rather than the use of virial coefficient data (which is admittedly scarce) to correct for gas-phase non-ideal behavior. As will be shown, these effects are in some cases very large, and must be dealt with when physico-chemical measurements are made by GC.

2. THEORY OF THE DETERMINATION OF VIRIAL COEFFICIENTS BY GAS–LIQUID CHROMATOGRAPHY

In 1961, Everett and Stoddart[4] reported the determination of activity coefficients for several hydrocarbon solutes in di-*n*-nonyl phthalate at 30° by gas–liquid chromatography (GLC). They also noted that the true activity coefficient for a single component when converted to fugacity, is only opproximately given by:

$$\ln \gamma_f^{\infty} = \ln \gamma_p^{\infty} - \frac{B\,p_1^0}{RT} \tag{12}$$

and, for an infinitely dilute solute vapor in a carrier gas, is actually:

$$\ln \gamma_f^{\infty} = \ln \gamma_p^{\infty} - \frac{p_1^0}{RT}\,(B_{11} - v_1^0) + \frac{\bar{p}}{RT}\,(2\,B_{12} - B_{22} - v_1^{\infty}) \tag{13}$$

where p_1^0 is the pure solute vapor pressure, v_1^0 the pure solute molar volume, $\bar{p}$ is the average column pressure, and v_1^{∞} is the solute molar volume at infinite dilution. In 1962, Goldup and co-workers[5] noted that the separation of several hydrocarbons could be dramatically altered merely by changing the carrier gas. The suggestion was made that second-interaction virial coefficients (B_{12} values) were responsible, and an empirical formula was proposed to account for the behavior:

$$\ln k' = A + \frac{2\,\bar{p}\,B_{12}}{RT} \tag{14}$$

where $k' = K_L V_L / V_G$, the capacity factor, and A is an empirical constant. At constant T and $\bar{p}$, $\ln k'$ was indeed shown to be a linear function of B_{12} for methylcyclopentane, 2,2-dimethylpentane, 2,4-dimethylpentane, and benzene, with helium, hydrogen, nitrogen, argon, and carbon dioxide carrier gases.

Desty and co-workers[6] evaluated the use of capillary columns in 1962, and concluded that B_{12} values could be determined via the following equation:

$$\ln V_N = \ln V_N^0 + \beta p_o J_2^3 \tag{15}$$

where V_N is the solute net retention volume, and:

$$\ln V_N^0 = \ln \frac{n_L RT}{\gamma_1^\infty p_1^0} - \frac{(B_{11} - v_1^0) p_1^0}{RT}$$

$$\beta = \frac{2 B_{12} - v_1^\infty}{RT}$$

n_L is the number of moles of stationary phase in the column, p_o is the column outlet pressure, and

$$J_2^3 = \frac{2}{3} \left[\frac{(p_i/p_o)^3 - 1}{(p_i/p_o)^2 - 1}\right]$$

following the nomenclature of Everett[7]:

$$J_n^m = \frac{n}{m} \left[\frac{(p_i/p_o)^m - 1}{(p_i/p_o)^n - 1}\right]$$

Everett[7] also derived a virial equation which was different from Desty's. The ideal gas law was written as a summation:

$$PV = n_G RT + \frac{RT}{V^2} \sum_{ij} B_{ij} X_i X_j \tag{16}$$

where n_G is the total number of moles of gas phase, and X_i is the mole fraction of the ith component. Everett then derived the following expression for the activity coefficient:

$$\ln \gamma_1^\infty = \ln \gamma_1^{\infty,*} - \frac{(B_{11} - v_1^0) p_1^0}{RT} + \frac{(2B_{12} - B_{22} - v_1^\infty) \bar{p}}{RT} \tag{17}$$

where

$$\gamma_1^{\infty,*} = \frac{n_L RT}{K_L V_L p_1^0} \left[1 + \frac{B_{22} \bar{p}}{RT}\right]$$

Thus,

$$\ln \gamma_1^\infty = \ln \frac{n_L RT}{K_L V_L p_1^0} - \frac{(B_{11} - v_1^0) p_1^0}{RT} + \frac{(2B_{12} - v_1^\infty) \bar{p}}{RT} \tag{18}$$

where the approximation:

$$\ln\left(1 + \frac{B_{22}\,\bar{p}}{RT}\right) \cong \frac{B_{22}\,\bar{p}}{RT}$$

has been made.

Eqn. 18 is formally similar to eqn. 15 by Desty. However, Everett subsequently derived the following:

$$\ln K_L = \ln K_L^0 + \beta\bar{p} \tag{19}$$

where

$$\ln K_L^0 = -\ln \gamma_1^\infty + \ln \frac{n_L\,RT}{V_L\,p_1^0} - \frac{(B_{11} - v_1^0)\,p_1^0}{RT}$$

and

$$\beta = \frac{(2B_{12} - v_1^\infty)}{RT}$$

as before. The net retention volume was then shown to be:

$$V_N = K_L^0\,V_L\,(1 + \beta\,p_o\,J_3^4) \tag{20}$$

Or, since $K_L^0 = V_N^0/V_L$,

$$K_L = K_L^0\,(1 + \beta\,p_o\,J_3^4) \tag{21}$$

A plot of V_N (or K_L) *vs.* $p_oJ_3^4$ should have a slope of $K_L^0V_L\beta$ (or $K_L^0\beta$), and an intercept of $K_L^0V_L$ (or K_L^0). The true activity coefficient should therefore be given by:

$$\ln \gamma_1^\infty = \ln \frac{n_L\,RT}{K_L^0\,V_L\,p_1^0} - \frac{(B_{11} - v_1^0)\,p_1^0}{RT} \tag{22}$$

Martire and Pollara[8] also considered expressions for the activity coefficient, and used the following:

$$\ln \gamma_1^\infty = \ln \gamma_p^\infty + \frac{(2B_{12} - v_1^0)\,\bar{p}}{RT} - \frac{(B_{11} - v_1^0)\,p_1^0}{RT} \tag{23}$$

where v_1^∞ has been replaced by v_1^0 in the second term on the right-hand side of eqn. 23. They noted that this equation should be used for all carrier gases except helium, which is nearly ideal, and for which the following approximation is probably valid to $\pm$ 1%:

$$\ln \gamma_1^\infty = \ln \gamma_p^\infty - \frac{p_1^0\,B_{11}}{RT} \tag{24}$$

Cruickshank *et al.*[9] and Windsor and Young[10] reconsidered the equations of

Desty and Everett. They also expanded the theory to include the effects of carrier gas solubility and third virial coefficients:

$$\ln V_N = \ln V_N^0 + \beta p_o J_3^4 + \xi (p_o J_3^4)^2 \tag{25}$$

where:

$$\xi = \frac{(3C_{122} - 4B_{12}B_{22})}{2(RT)^2} \tag{26}$$

C_{122} is a mixed third virial coefficient[11], and is probably negligible up to 20 atm. Eqn. 25 is only approximate if β is defined as before. More correctly, however[12,13],

$$\beta = \frac{(2B_{12} - v_1^\infty)}{RT} + \lambda \left[1 - \left(\frac{\partial \ln \gamma_1^\infty}{\partial X_2}\right)\right] \tag{27}$$

where λ is the carrier gas molal solubility in the stationary phase, X_2 is the carrier mole fraction in the stationary phase, and $(\partial \ln \gamma_1^\infty / \partial X_2)$ represents the change of the solute activity coefficient with changing amount of dissolved carrier. When the corrections given by eqns. 26 and 27 are ignored, eqn. 25 reduces to:

$$\ln V_N = \ln V_N^0 + \beta p_o J_3^4 \tag{28}$$

Three equations had thus been derived by different workers, eqns. 15, 20, and 28, which are given below in terms of the net retention volume:

$$\ln V_N = \ln V_N^0 + \beta p_o J_2^3 \qquad \text{(Desty } et\ al.^6\text{)} \tag{15}$$

$$\ln V_N = \ln V_N^0 + \ln (1 + \beta p_o J_3^4) \text{ (Everett}^7\text{)} \tag{20}$$

$$\ln V_N = \ln V_N^0 + \beta p_o J_3^4 \qquad \text{(Cruickshank } et\ al.^9\text{)} \tag{28}$$

Each of these involves a different plotting procedure to obtain β (and hence B_{12}). Cruickshank *et al.*[9] and Windsor and Young[10] compared the three equations by assuming a value for β, then calculating V_N for a range of p_i and p_o values. Each of the three plotting procedures given by eqns. 15, 20, and 28 above were then used to retrieve β; eqn. 28 consistently gave β values within 0.3% of the initially assumed value (even for conditions similar to capillary columns), and was usually much better than 0.3%. The only difference between eqns. 15 and 28 is in the J term, and results from either equation were not appreciably different as long as B_{12} was less than about 150 ml/mole.

Sewell and Stock[14] investigated the solubility of nitrogen in squalane and found it to be negligible. Cruickshank and co-workers[12,13] also considered the magnitude of the ξ and modified β terms given above. Neglecting ξ will give an error in the virial coefficient of about $\pm$ 2 ml/mole, but ignoring the term

$$\lambda \left[1 - \left(\frac{\partial \ln \gamma_1^\infty}{\partial X_2}\right)\right] \tag{29}$$

may lead to appreciable errors; for hydrocarbon solutes and stationary phases at

column pressures less than 5 atm, the B_{12} values will be erroneous by 3 ± 3 ml/mole (H_2 carrier), 6 ± 6 ml/mole (N_2 carrier), and 10 ± 10 ml/mole (Ar carrier). The carrier effects will be even larger at pressures greater than 5 atm due to the increased solubility of the carrier in the stationary phase.

Pecsok and Windsor[15] extended the study of carrier gas effects to include methane and ethane, with the use of a very sensitive katharometer[16]. Their equation was of the form:

$$\ln V_N \left[\frac{(1 + b\bar{p})}{(1 + bp_o)}\right] = \ln V_N^0 + \beta p_o J_3^4 \tag{30}$$

where $b = B_{22}/RT$, $\bar{p} = p_o J_2^3$, and β is given by eqn. 27 above. The pressure drop in their work was very small ($\bar{p} - p_o < 0.1$ atm), so that $(1 + b\bar{p})/(1 + bp_o)$ was close to unity; the discrepancy in the data with ethane as a carrier was only 2 ml/mole when the factor was ignored. The carrier solubility was expected to be appreciable, since the stationary phase was squalane. Virtually no data exist for the value of $\{1 - [(\partial \ln \gamma_1^\infty)/(\partial X_2)]\}$, however, and so the approximation was made that it lies between 0 and 1, that is:

$$\left[1 - \left(\frac{\partial \ln \gamma_1^\infty}{\partial X_2}\right)\right] = 0.5 \pm 0.5$$

A further approximation was the assumption that the solubility behavior of the carrier gases was ideal[17]. The corrections to B_{12} values, when the term given by eqn. 29 is included in eqn. 27, were -22 ± 22 ml/mole for methane carrier, and -153 ± 153 ml/mole for ethane carrier at 25°. These were very approximate, however, and indicated that much more accurate determinations of λ and $[(\partial \ln \gamma_1^\infty)/(\partial X_2)]$ are required when hydrocarbons are used as carrier gases.

Dantzler *et al.*[18] critically compared static[19] and GLC-determined B_{12} values, and found that the agreement was within the experimental error of the two techniques. They also noted that replacing v_1^∞ by v_1^0 in eqn. 27 may give an error of 10 ml/mole in the B_{12} values determined by GLC. Ignoring carrier gas solubility (when fixed gases are used) may additionally cause an error of $\pm 5 - 10$ ml/mole, so that while the precision of GLC experiments can be as good as ± 6 ml/mole, the overall error of the method may be as high as $\pm 20 - 30$ ml/mole. Cruickshank *et al.*[20] attempted to avoid the carrier solubility problem by using a polar stationary phase (glycerol), where the solubility of N_2 and CO_2 was estimated to be less than 10% of the solubility of these gases in hydrocarbon phases. Gainey and Pecsok[21] used a series of closely related stationary phases for a number of hydrocarbon solutes and nitrogen carrier, and found the agreement between GLC and calculated values was excellent when N_2 solubility in the stationary phases was taken into account.

Vigdergauz and Semkin[22] have reported the determination of B_{12} values from the change of retention index with pressure:

$$\frac{\Delta I}{\Delta P} = \frac{(I_1 - 100\,Z)\,(\beta_{Z+1} - \beta_Z) - 100\,(\beta_X - \beta_Z)}{-b_2} \tag{31}$$

where $\Delta I = I_2 - I_1$ (the retention indices of the solute of interest measured at P_2

and P_1), Z and $Z + 1$ are the carbon numbers of the standards, $\Delta P = P_2 - P_1$, X is the solute of interest, and:

$$\beta_i = \frac{2B_{12,i} - v_i^\infty}{2.303\,RT}$$

$$b_2 = \log \frac{V_{g,Z+1}}{V_{g,Z}}$$

where b_2 is the log of the ratio of specific retention volumes of the standards at P_2. In addition, they described a capillary column method which used benzene and styrene standards, PEG 400 as the stationary phase, and pressures up to 30 atm for the determination of B_{12} values for several aromatic hydrocarbons. A method was also proposed which allows calculation of the $[(\partial \ln \gamma_1^\infty)/(\partial X_2)]$ term by choosing one of the solutes as a standard. Finally, Spertell and Chang[23] have derived a method of determining solute–solute (B_{11}) virial coefficients by GLC, where the solute is an isotope of the carrier gas; the proposed method appears to be valid, but is severely limited by the isotopic requirements of the solute, and has not yet been experimentally verified.

3. EXPERIMENTAL ASPECTS

A. Apparatus

The apparatus requirements for the determination of virial coefficients by GLC are essentially the same as for any physico-chemical measurement[24], with the added consideration that the pressure must be variable over a range of several atmospheres. Many authors have reported GC apparatus capable of medium-to-high pressure operation, including Young[25], Pecsok and Windsor[15,16], Tsuda *et al.*[26], and Cruickshank *et al.*[27,28]. Goedert and Guiochon[29,30] have also described a high-precision apparatus capable of reproducing retention times to hundredths of a second. The device described by Tsuda *et al.*[26] is of particular interest, since organic solvents such as carbon tetrachloride, benzene, and ethanol were used as carrier gases. These of course exhibit large gas-phase non-ideal effects, but may prove useful in the separation of some components, especially in light of the findings of Goldup and co-workers[5], where the elution behavior of petroleum hydrocarbons was markedly altered merely by changing the carrier gas.

Two excellent papers have also appeared which describe useful apparatus for the static measurement of B_{12} values. Coan and King's method[31] used an entrainment procedure to determine the mole fraction of benzene in various gases over a range of 40 atm. The mixed virial coefficients were then found from the ratio of fugacity coefficients of benzene and the benzene–gas mixtures. Knobler[19] has also described a device which was employed by Dantzler *et al.*[18] in order to compare GLC and static B_{12} values.

B. Calculated B_{12} values

Good agreement has been obtained between virial coefficients calculated from molecular properties, and those determined by GLC. Cruickshank and co-workers[12,32]

first reported the use of the "method of corresponding states", due to Hudson and McCoubrey[33]. The principle of corresponding states requires that if two different gases have the same value for two reduced variables (*e.g.*, pressure and temperature), they will also have approximately the same value for the third reduced variable (*e.g.*, volume), and are said to be in corresponding states[34]. This principle has been used by several workers to calculate B_{12} values, notably McGlashan *et al.*[35,36], and Guggenheim *et al.*[37,38]. Gainey and Hicks[39,40] have recently reviewed four methods of predicting B_{12} values, and it now appears that solute mixed virial coefficients can be readily calculated, even with a paucity of experimental information regarding the molecular properties of the compounds of interest.

C. GLC procedure

Mixed virial coefficients are determined most accurately by GLC with eqn. 28: $\ln V_N$ is plotted *vs.* $p_o J_3^4$, which should give a straight line of slope β. B_{12} values are then found from this slope and eqn. 27; several examples are given by Littlewood[41], where plots of $\log V_N$ *vs.* $p_o J_3^4$ are presented for benzene in squalane with various carrier gases (taken from ref. 28). Helium gives almost a horizontal line, indicating that virial effects are negligible up to several atmospheres. The heavier carrier gases, however, show appreciable (greater than 1%) virial deviation from ideal behavior beyond 2 atm. Values for the second-interaction virial coefficients determined in this way will not be more accurate than $\pm$ 20 ml/mole, however, unless the effects of carrier solubility in the stationary phase are known, or can be negated[22]. In addition, the average column pressure should be kept below approximately 10 atm so that third virial coefficients (C_{ijk}) are negligible (eqn. 26). Interestingly enough, Czubryt *et al.*[42] have shown that gas–solid chromatography is not a suitable technique for the determination of B_{12} values unless the carrier gas adsorption isotherm at the column temperature is known exactly, and the relative amounts of adsorbed carrier and solute on the packing surface can be determined.

B_{12} values are most often employed to calculate corrected (fugacity) activity coefficients; the use of eqns. 13, 23, or the following by Conder and Purnell[43]:

$$\ln \gamma_1^{\infty} = \ln \frac{n_L RT}{K_L V_L p_1^0} + \frac{(B_{11} - v_1^0) p_1^0}{RT} + \frac{(2B_{12} - v_1^{\infty}) p_o J_3^4}{RT} \qquad (32)$$

requires knowledge of the solute virial coefficient, B_{11}, in addition to B_{12} values. B_{11} values have been calculated by various methods, including that by Rowlinson[44], Guggenheim and McGlashan[37], Kobe and Lynn[45], Hirschfelder *et al.*[46], and McGlashan and Potter[35]. A simplified apparatus has recently been described with which B_{11} values can be determined directly[47]; a few examples are presented in Table 1.

Vapor pressure data (p_1^0 values) are also needed, and can usually be found in physical properties compendia, or calculated from various forms of the Antoine equation[48]. Solute molar volumes can easily be measured, but infinite-dilution molar volumes (v_1^{∞}) generally must be approximated (often, merely by substituting v_1^0); in selected cases, v_1^{∞} values can be calculated with some degree of accuracy[12,49].

TABLE 1

SOLUTE–SOLUTE VIRIAL COEFFICIENTS[6,41] (B_{11}) AT 25°

Solute	B_{11} *(ml/mole)*
n-Pentane	−1033
n-Hexane	−1468
n-Heptane	−1968
Benzene	−1326
Cyclohexane	−1510

4. RESULTS

At least 17 papers have now appeared which list B_{12} values for well over 200 organic compounds with various carrier gases and stationary phases. A few representative examples are given in Tables 2 and 3: Table 2 compares values of pentane for various carriers and stationary phases, and Table 3 presents the virial coefficients for a variety of hydrocarbons. Most of the B_{12} determinations have been for normal and branched alkanes and alkenes, but a few aromatic hydrocarbons have also been studied[22].

TABLE 2

COMPARISON OF B_{12} VALUES FOR PENTANE

Solvent	*Carrier*	*T (°C)*	B_{12} *(ml/mole)*	*Reference*
Squalane	N_2	25	−100	27
Squalane	H_2	25	+ 1	27
Squalane	N_2	25	− 76	7
Squalane	H_2	25	+ 3	7
Squalane	He	25	+ 28	6
Squalane	CH_4	25	−204	15
Squalane	C_2H_6	25	−414	15
n-Hydrocarbons	Ar	25	− 98	18
1-Phenylalkanes	N_2	40	− 86	21
n-Octadecane	N_2	35	− 85	12
Di-*n*-nonyl phthalate	Ar	50	− 82	22
Di-*n*-nonyl phthalate	Ar	80	− 68	22
Di-*n*-nonyl phthalate	N_2	80	− 60	22
Di-*n*-nonyl phthalate	CO_2	80	− 76	22

A. Comparison of the GLC technique with other methods

Chromatographic data are usually evaluated by comparing GLC B_{12} values to static measurements, or calculated virial coefficients. The B_{12} values for benzene determined by the static method of Coan and King[31] are compared to GLC measurements in Table 4; agreement between the two techniques is within the experimental errors of the methods (Table 2, ref. 31). Dantzler *et al.*[18] also found that agreement between their static and GLC B_{12} values was within experimental error, which was said to be as high as ± 20–30 ml/mole. This is not overly distressing, since at $\bar{p} = 1$ atm, a B_{12} value of ± 50 ml/mole will yield an activity coefficient accurate to ± 0.4%, as shown by Conder and Purnell[43].

TABLE 3

SELECTED VIRIAL COEFFICIENTS FOR HYDROCARBONS DETERMINED BY GLC

Solute	*Solvent*	*Carrier*	*T (°C)*	B_{12} *(ml/mole)*	*Reference*
n-Hexane	1-Phenylalkanes	N_2	40	−110	21
n-Heptane	1-Phenylalkanes	N_2	40	−110	21
n-Octane	1-Phenylalkanes	N_2	40	−134	21
n-Hexane	Squalane	CH_4	25	−292	15
2-Methylpentane	Squalane	CH_4	25	−317	15
2,2-Dimethylbutane	Squalane	CH_4	25	−216	15
Cyclohexane	*n*-Octadecane	N_2	35	−122	12
Benzene	*n*-Octadecane	N_2	35	−104	12
Hexafluorobenzene	1-Phenylalkanes	N_2	40	−126	21
Toluene	Polyethylene glycol 400	CO_2	50	−248	22
Ethylbenzene	Polyethylene glycol 400	CO_2	50	−271	22
o-Xylene	Polyethylene glycol 400	CO_2	50	−289	22
m-Xylene	Polyethylene glycol 400	CO_2	50	−282	22
p-Xylene	Polyethylene glycol 400	CO_2	50	−284	22

In addition to the static experimental methods already cited, good agreement has also been found between calculated and GLC virial coefficients, as noted earlier. Conder and Langer[50], Gainey and Young[13], Gainey and Pecsok[21], and Gainey and Hicks[39,40] have all shown that the method of McGlashan and Potter[35] in conjunction with the combining rule of Hudson and McCoubrey[33] gives the best theoretical prediction of virial coefficients for conditions appropriate to GLC, and that values calculated in this manner agree with GLC results to 1–10%, as shown in Table 5. Thus, it appears that the GLC method of B_{12} measurement is accurate to about ± 10–20 ml/mole, and can be significantly better, providing the carrier solubility and third virial coefficient effects can be determined, or experimental procedures chosen so that they can be neglected. In any event, agreement between static, calculated, and GLC mixed virial coefficients lies within the experimental error of the respective methods, but the GLC technique is significantly faster and simpler, since it requires only a gas chromatograph and the determination of the solute net retention volume at several column pressures.

TABLE 4

COMPARISON OF STATIC AND GLC-DETERMINED B_{12} VALUES FOR BENZENE

Carrier gas	*T (°C)*	B_{12} *(ml/mole)*	
		Static[31]	*GLC*[15,17]
He	50	+ 67 ± 4	+ 57 ± 8
H_2	50	+ 4 ± 3	− 5 ± 8
N_2	35	− 97 ± 3	−104 ± 10
N_2	50	− 85 ± 3	− 87 ± 8
Ar	32	−122 ± 3	−135 ± 10
Ar	50	− 95 ± 3	− 85 ± 8
CH_4	50	−171 ± 3	−155 ± 15
C_2H_4	50	−282 ± 5	−

TABLE 5

CALCULATED AND GLC B_{12} VALUES FOR BENZENE–NITROGEN[13]

Solvent	*T (°C)*	B_{12} *(ml/mole)*	
		GLC	*Calc.*
n-Hexadecane	20	-120 ± 12	-120
	25	-109 ± 10	-116
	30	-107 ± 10	-111
n-Octadecane	35	-104 ± 10	-107
n-Eicosane	50	-94 ± 10	-96
	60	-93 ± 10	-89

5. DISCUSSION

There are two areas of significance for chromatographers arising from virial coefficient studies, the first is the effect of gas-phase non-ideal behavior on GC data, and the second, the use of organic solvents as carrier gases. Virial effects are now recognized as a potential source of significant error in GC results, but these gas imperfections may in some cases be used to separate components. Each of these topics is therefore now examined in detail.

A. Virial effects on V_g and K_L values

Littlewood[41] has considered the problem of non-ideal (virial) effects and relative distribution coefficients. He defined the distribution coefficient, K_L, in terms of mass:

$$K_L = \frac{\text{weight of solute per gram stationary phase}}{\text{weight of solute per milliliter carrier gas}}, \text{ ml/g}$$

and showed that:

$$K_L = \frac{RT}{\gamma_1^\infty MW_L p_1^0}, \text{ ml/g}$$

where MW_L is the molecular weight of the liquid phase. The true distribution coefficient, K_L' (corrected for carrier non-ideal behavior), was then given as:

$$K_L' = K_L \exp\left[\frac{(v_1^0 - B_{11})\,p_1^0}{RT} + \frac{(2B_{12} - v_1^\infty)\,\bar{p}}{RT}\right] \tag{33}$$

The ratio of K_L' to K_L will thus be a measure of virial effects on the distribution coefficients:

$$\ln \frac{K_L'}{K_L} = \frac{(v_1^0 - B_{11})\,p_1^0}{RT} + \frac{(2B_{12} - v_1^\infty)\,\bar{p}}{RT} \tag{34}$$

Cruickshank and co-workers[9] have correctly pointed out that even if $B_{12} = 0$, the term $(-v_1^\infty/RT)$ is not zero under any conceivable GLC conditions, so that the ratio will always differ from unity, even at $p_o J_3^4 = 0$, at which point:

$$\ln \frac{K_L'}{K_L} = \frac{(v_1^0 - B_{11})\,p_1^0}{RT} \tag{35}$$

Conder and Langer[50] have also considered the effects of gas-phase non-ideal behavior on retention volumes. They showed that:

$$\ln \frac{V_g\,(\mathrm{I})}{V_g\,(\mathrm{II})} = \frac{2\,p_o\,J_3^4}{RT}\,[B_{12}\,(\mathrm{I}) - B_{12}\,(\mathrm{II})] \tag{36}$$

where $V_g(i)$ and $B_{12}(i)$ are the specific retention volume and virial coefficient for carrier gas, i, with the same liquid phase. Eqn. 36 predicts that the ratio of specific retention volumes is independent of B_{11} or B_{22} values, and the effect of changing the carrier gas (assuming it is insoluble) is independent of the stationary phase. This was demonstrated to be the case for butyl tetrachlorophthalate and benzoquinoline. Thus, while the absolute specific retention volumes were appreciably different for the two phases, the ratio $\ln [V_g\,(\mathrm{I})]/[V_g\,(\mathrm{II})]$ was the same for both phases. Conder and Langer[50] also found that the difference in V_g values was 1–2% for a variety of aliphatic and aromatic hydrocarbons when helium and nitrogen were compared as carrier gases, which is certainly negligible for most analytical packed-column work (especially since commercially available chromatographs are rarely capable of reproducing and controlling column temperature and flow-rate to better than $\pm$ 5%). However, they also noted that since capillary columns often require an appreciable pressure drop across long narrow-bore tubing to maintain an adequate flow-rate, virial effects can become appreciable, which will yield non-reproducible relative retention volumes as well as absolute values, unless the pressure conditions are duplicated exactly from laboratory to laboratory. This of course applies equally well to packed-column work whenever the pressure drop across the column exceeds 2 atm. These contributing factors to the non-reproducibility of GC data are not recognized by most chromatographers; and, while it is often true that most analytical laboratories are not concerned with reproducibility of better than 5–10%, such inaccuracy can lead (for example) to the incorrect identification of compounds in complex mixtures, for which capillary columns are now primarily used, and for which virial effects may be pronounced.

B. Use of organic carrier gases

The use of carrier gases such as benzene[26] may prove very useful in the separation of mixtures, since B_{12} values should be large. For example, Laub and Pecsok[51,52], and Purnell and co-workers[53,54] have recently examined the charge transfer interactions of benzene, toluene, and the xylenes with various complexing agents in a variety of stationary phases. A very interesting experiment would be the use of benzene as a carrier gas with a column containing di-*n*-butyl phthalate and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ; a strong complexing agent[51]). Presumably, the stationary phase would quickly become saturated with benzene, which would then complex with the dissolved DDQ. Solutes such as toluene and the xylenes would be forced to compete with benzene for sites of complexation, thus offering an additional column variable for purposes of separation, similar to the competition between carrier gases and solutes for surface sites in gas–solid chromatography. Alternatively, one could conceivably begin with a carrier of *p*-xylene (or various dilutions thereof in fixed gases) which forms stronger complexes than the other xylenes, and attempt the elution of the aromatic hydrocarbons. It is anticipated that frontal or displacement forms of GLC would also prove useful in these studies. Luckhurst[55], and Conder

and Purnell[43] have in fact considered the determination of activity and virial coefficients when the solute concentration is not at infinite dilution, and Conder[24] has pointed out the advantages of several finite-concentration GLC methods.

The technique of Coan and King[31] would appear to be particularly useful as an independent measure of B_{12} values where an organic carrier gas is used, such as ethane or benzene. Of perhaps potentially greater importance, however, would be a comparison of the static values to the GLC virial coefficients in order to determine the effects of carrier gas solubility in the stationary phase. It may also be feasible to use a combined McBain balance[56]–DeNouy tensiometer[57] system to measure carrier solubility directly, analogous to the determination of GLC surface adsorption effects[58–63]. Regardless of the procedure or measurement, however, the use of carrier gases other than helium, hydrogen, or nitrogen is a much neglected area of research, and it seems feasible that carrier selectivity in GC could become as important as solvent elution in high-performance liquid chromatography.

6. CONCLUSIONS

It should now be abundantly clear that virial effects can be very important in physico-chemical studies by GLC, particularly when activity coefficients are to be determined. As discussed earlier, the error in γ_1^∞ values will be as much as 5–10% if virial effects are ignored, and it is not difficult to realize that since chromatographic distribution phenomena depend inversely on γ_1^∞, data from non-physico-chemical applications (such as separation and identification) will similarly be in error. These effects will be most pronounced when large pressure drops across a column are used, resulting in the possibility of serious discrepancies in retention data. The alteration of retention times or volumes by changing the carrier gas, however, may be a very useful separation tool for analytical work.

An excellent example of other virial effects can be found in the work reported by Yeramian *et al.*[64], who studied the influence of "inert" diluent gases on the reaction rate and activation energy of the oxidation of SO_2 to SO_3 with V_2O_5 catalyst at 375–450°. Arrhenius plots showed that there was a 100% difference in the reaction rate when helium was substituted for argon as the diluent gas, and an increase in the molecular weight of the gas increased the rate, precisely the opposite of what was expected. Furthermore, adsorption of the inert gas on the surface of the catalyst was discounted as minimal at the temperature of interest (although this may be open to question), so that physico- or chemi-sorption could not be used to explain the anomalous rate behavior; these phenomena have yet to be explained, but are of considerable interest in a wide variety of industrial applications, and therefore continue to be studied in detail[65]. Such investigations are of course directly amenable to the GLC or GSC methods developed for rate and catalysis studies[66]; for example, a glance at Tables 2 and 4 shows that He and Ar give virial interactions which are different by as much as 160 ml/mole (benzene at 50°). A useful approach to the question of SO_2 catalysis would therefore involve the determination of the virial coefficients of He and Ar with SO_2 (undoubtedly, these effects are pronounced with species such as SO_2). Carrier gas effects could then subsequently be examined by GSC, where V_2O_5 would be employed as the column packing.

Finally, it is now apparent to chemists of all disciplines that gas–liquid chro-

matography is of far more importance than a mere tool for separations. Nowhere is this more true than in physical chemistry, where GLC is becoming an increasingly attractive method of examining solution phenomena and related topics[17,51–54,67–71]. Concerning virial phenomena, the trend in GLC has for the most part been in the direction of determining B_{12} values, rather than the use of virial coefficients to obtain more accurate (hence more reproducible) distribution data. Yet, it does little good to construct a high-precision instrument, and not correct the data so obtained for carrier gas non-ideal effects. It is hoped, therefore, that this review will prompt chromatographers at least to consider virial phenomena, not only in physico-chemical applications, but in straightforward separations as well. There are many areas awaiting development in the study of virial imperfections, not the least of which is the use of common organic solvents as GC carrier gases. Lastly, this review should serve to indicate the fulfillment of an expectation by Purnell[72], who in 1962 expressed confidence that GC would one day assume an established place among purely physico-chemical disciplines.

7. SUMMARY

The determination of second-interaction virial coefficients (B_{12} values) by gas–liquid chromatography is reviewed. The precision apparatus and experimental procedures required to measure B_{12} values are considered, and the importance of virial coefficients is also discussed. It is shown that in some cases, carrier gas imperfections lead to very pronounced solute elution effects, especially in kinetic studies. It is concluded that virial coefficients should always be taken into account whenever physico-chemical measurements are made by gas chromatography, and that virial effects should also be recognized to be of potential importance in other analytical (separation and identification) studies.

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Chromatographic Reviews

CHREV. 80

CHROMATOGRAPHY OF THE 1,4-BENZODIAZEPINES

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1. INTRODUCTION

Since their introduction into clinical use in the early 1960s, the 1,4-benzodiazepine drugs have established themselves as widely used tranquillizers, sleep inducers and muscle relaxants. With the extensive use of these compounds there has been a parallel evolution of analytical methods for their determination during development and in clinical and forensic situations. From the point of view of analysis in biological, especially human, samples, there are two important considerations. Firstly, the levels of the drugs are low, and secondly their chemically similar metabolites may also be pharmacologically active, and thus also require determination. These ana-

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lytical requirements have led to the widespread use of chromatographic techniques in the determination of the benzodiazepines. This review covers the progress in the chromatographic analysis of those benzodiazepines which are marketed or have had extensive clinical trials. It is likely that many more compounds of this type will come into clinical use in the future. The work reviewed here should provide a basis for further analytical work.

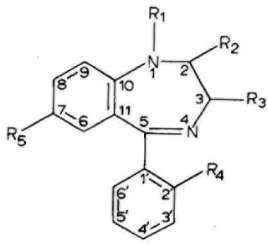


Fig. 1. General formula of the 1,4-benzodiazepines.

The general formula of the 1,4-benzodiazepines is shown in Fig. 1. The substituent at R_5 is invariably a halogen atom or a nitro group in the compounds considered here, an electronegative substituent in this position being essential for useful pharmacological activity[1]. The two main metabolic routes for the benzodiazepines involve dealkylation of the N_1 atom, and hydroxylation at C_3. In addition, the 7-nitro compounds are metabolized by reduction to the 7-amino- and 7-acetamidobenzodiazepines. Hydroxylation of the 4′-position is an important process in several animals. The benzodiazepines are eliminated from the body mainly as the glucuronide and sulphate conjugates of the hydroxy and 7-amino metabolites. Metabolism of the benzodiazepines has been reviewed by Schwartz[2] and by Garrattini *et al.*[3].

Many benzodiazepines are hydrolyzed by strong acids to give benzophenones. These hydrolysis products have frequently been used in chromatographic analysis, and are listed in Table 1. It will be seen that certain benzophenones are formed by more than one benzodiazepine.

As is the case with other drugs, analytical support for the benzodiazepines is required in four areas:

(1) During development of the drugs, in elucidating their metabolism and studying their toxicology.

(2) In checking for purity and specification in the manufacture of the drug.

(3) In studying the pharmacokinetics and obtaining tissue and body fluid levels in clinical use.

(4) In obtaining tissue and body fluid levels in forensic situations, often following overdosage.

In the case of the benzodiazepines, chromatographic techniques have been widely used in all four areas. Each of these has its own requirements, and chromatographic methods developed for one field are not necessarily applicable in another. In the development of a drug, identification is often facilitated by the use of radioactively labelled material, which is obviously not feasible in the other types of analysis. Chromatographic analysis of benzodiazepines for quality control and forensic

TABLE 1

HYDROLYSIS PRODUCTS OF SOME BENZODIAZEPINES

Benzodiazepine	*Hydrolysis product*	*Abbreviation*
Chlordiazepoxide Desmethylchlordiazepoxide Demoxepam Desmethyldiazepam Oxazepam Chlorazepate Oxazolam	2-Amino-5-chlorobenzophenone	ACB
Diazepam 3-Hydroxydiazepam	2-Methylamino-5-chlorobenzophenone	MACB
Cloxazolam	2-Amino-7,2′-dichlorobenzophenone	ACCB
Nitrazepam	2-Amino-5-nitrobenzophenone	ANB
7-Aminonitrazepam 7-Acetamidonitrazepam	2,5-Diaminobenzophenone	DAB
Clonazepam	2-Amino-2′-chloro-5-nitrobenzophenone	ANCB
7-Aminoclonazepam 7-Acetamidoclonazepam	2,5-Diamino-2′-chlorobenzophenone	DACB
Bromazepam 3-Hydroxybromazepam	2-Amino-5-bromobenzoylpyridine	ABBP
Flurazepam 3-Hydroxyflurazepam	2-Ethyldiethylamino-5-chloro-2′-fluorobenzophenone	
N-Desalkylflurazepam	2-Amino-5-chloro-2′-fluorobenzophenone	ACFB
Flunitrazepam	2-Methylamino-5-nitro-2′-fluorobenzophenone	MANFB
Desmethylflunitrazepam	2-Amino-5-nitro-2′-fluorobenzophenone	ANFB

purposes involves detection of the drugs in relatively high concentrations, and methods used for these purposes are often inadequate for situations following therapeutic administration of the drugs. Blood levels of the benzodiazepines following therapeutic dosage are frequently in the range of 10–500 ng/ml. Chromatographic methods suitable for the drugs and their metabolites at this level in some cases require taking chromatographic techniques to their limits of sensitivity. This review emphasizes in particular the chromatographic analysis of the benzodiazepines in body fluids.

Extraction of the benzodiazepines from biological material has been described using a variety of solvent systems. The compounds have frequently been extracted from samples buffered to pH 9. As many benzodiazepines are strongly protein bound[4,5], vigorous shaking during the extraction step has been recommended by some workers[6]. When dealing with very low levels of the compounds, an acid clean-up stage to remove lipids and other interfering substances is often performed. Extraction solvents must be free of co-chromatographable impurities. In the case of urinary metabolites, chromatography of the intact compounds is normally carried out following hydrolysis of the conjugates by β-glucuronidase.

Gas–liquid chromatography (GLC) has been used extensively in the analysis of the benzodiazepines. It has often been possible to chromatograph the compounds intact, without derivatisation, especially at the microgram level. Chromatography at

low levels, such as those found in blood following a single therapeutic dose, is more demanding. At the nanogram level, adsorption processes become significant, especially for the N-desalkyl compounds, and processes such as photolytic decomposition may also be important. Partly for these reasons, a number of GLC methods involve chromatography of the benzophenone hydrolysis products rather than the benzodiazepines themselves. Determination of low levels of benzodiazepines or their hydrolysis products by GLC has often necessitated the use of electron capture detection. This in turn has implied injection of moisture free extracts onto the column, and use of clean-up procedures or selective extraction to minimize contamination of the detector[6,7].

Thin-layer chromatography (TLC) has been used for all of the benzodiazepines. Separations have most often been carried out on silica gel plates, often using solvent systems based on chloroform or ethyl acetate. Two-dimensional separations have been especially useful in metabolic studies. TLC of the hydrolysis products has frequently been used in toxicological and forensic work, sometimes resulting in reduced specificity. Detection is commonly achieved with modified Dragendorff reagent, platinum–iodine reagent, Bratton–Marshall derivatization in the case of compounds with primary amino groups, fluorescence quenching, and conversion to fluorophores (*e.g.*, acridones) with concentrated acids.

2. CHROMATOGRAPHY OF THE BENZODIAZEPINES IN METABOLIC STUDIES AND CLINICAL PHARMACOLOGY

A. Diazepam, medazepam and their metabolites

(a) Diazepam

Diazepam and its metabolites have been studied more intensively than the other benzodiazepines. All three major metabolites (Fig. 2) have pharmacological activity. Oxazepam is marketed separately as a tranquilliser, and desmethyldiazepam (Ro 5-2180) and temazepam (Ro 5-5345) have both undergone clinical trials, and are likely to be made available for general medical use.

Gas chromatography has been used extensively in the determination of diazepam and metabolites in body fluids (Table 2), and the evolution of techniques from chromatography of the hydrolyzed products (benzophenones) to separation of the intact benzodiazepines reflects the introduction of improved materials for gas chromatography of low levels of compounds. It was quickly realised that these compounds were very suitable for electron capture gas chromatography, mainly because of the presence of the electronegative substituent at C_7. De Silva *et al.*[8], in the first gas chromatographic method to be published, hydrolyzed diazepam and its metabolites with 6 *N* hydrochloric acid to give the benzophenones ACB and MACB following extraction of plasma samples with diethyl ether. These derivatives were more volatile than the benzodiazepines and their chromatography was easier. A 2% Carbowax 20M stationary phase on silanized Gas-Chrom P was used in conjunction with a tritium electron capture detector (Fig. 3). Linear response to MACB and good reproducibility were obtained in a new column, but column performance deteriorated with age, and the average useful life was only 2 weeks. The method was later modified[9] by using a liquid phase of 2% Carbowax 20M–terephthalic acid[10].

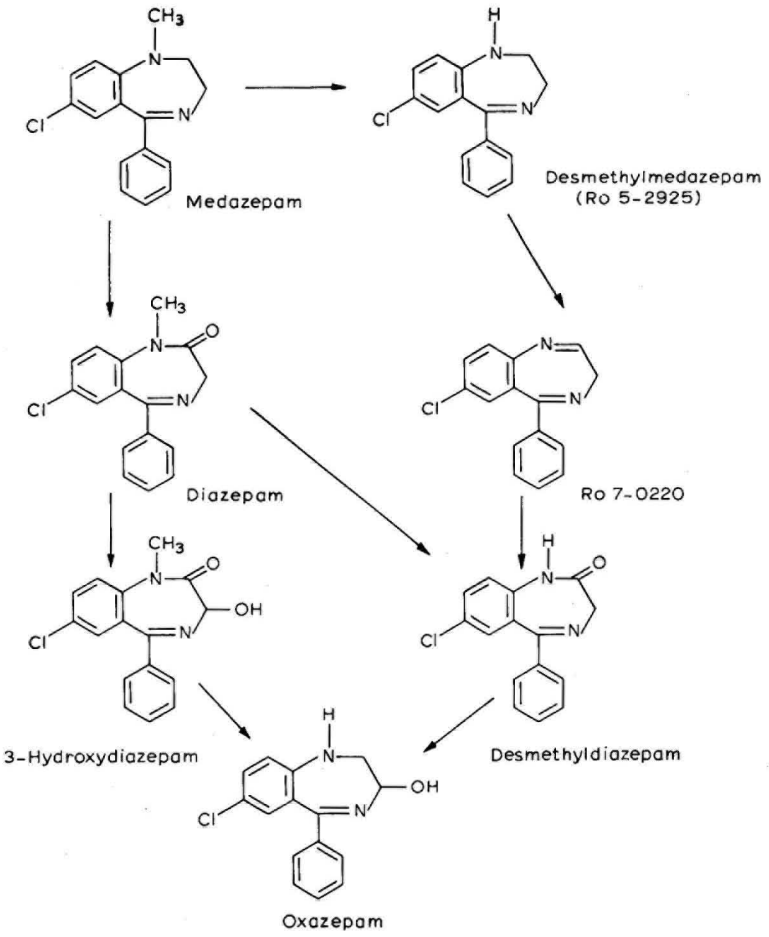


Fig. 2. Metabolic pathways for medazepam and diazepam.

This more polar phase had greater temperature stability, better coating characteristics and longer column life.

A very similar method was subsequently published by Cano *et al.*[11,12]. All these methods based on chromatography of the benzophenones suffered from a lack of specificity, as hydrolysis of diazepam and its three metabolites gives rise to only two benzophenones. It is therefore not possible to differentiate 3-hydroxydiazepam from diazepam, or oxazepam from desmethyldiazepam. In practice, this may not be a major shortcoming in the analysis of blood samples, as only diazepam and its desmethyl metabolite are present in significant amounts in the circulation following a single therapeutic dose or short-term administration. In addition, there were problems regarding co-extracted compounds which gave peaks at or close to the retention volumes of the benzophenones. This point was considered in some detail in De Silva *et al.*'s original paper[8]. Washing the acid phase with ether prior to hydrolysis gave cleaner chromatograms than clean-up of ether extracts or acid with various

TABLE 2

GLC METHODS FOR DIAZEPAM, MEDAZEPAM AND THEIR METABOLITES

Workers	*Column*	*Column temperature (°C)*	*Detector*	*Internal standard*	*Extraction solvent*	*Remarks*
De Silva *et al.*[8] (1964)	2-ft. stainless steel, 2% Carbowax 20M	190	Tritium ECD	—	Diethyl ether	Hydrolysis to ACB and MACB prior to chromatography
De Silva *et al.*[9] (1966)	2-ft. stainless steel, 2% Carbowax 20M–TPA on Gas-Chrom P, 100–120 mesh	215	Tritium ECD	—	Diethyl ether	As above
Cano *et al.*[11] (1967)	2-m stainless steel, 2% Carbowax 20M on Chromosorb	195	Tritium ECD	—	Diethyl ether	As above
Marcucci *et al.*[13] (1968)	2-m glass, 3% OV-1 on Gas-Chrom Q, 60–80 mesh	245	FID	BACB	Diethyl ether	Intact benzodiazepines, relatively high concentrations
De Silva and Puglisi[6] (1970)	4-ft. glass, 3% OV-17 on Gas-Chrom Q, 60–80 mesh	235	^{63}Ni ECD	Medazepam	Diethyl ether, followed by acid clean-up	Method for both intact diazepam and medazepam. No internal standard when used for medazepam
Foster and Frings[19] (1970)	2-m × 2-mm I.D. glass, 3% SE-30 on Chromosorb W, 80–100 mesh	205	FID	—	Chloroform	Detection of metabolites not reported. Suitable for overdose or chronic medication situations
Van der Kleijn *et al.*[15] (1971)	1-m glass, 3% OV-17 on Gas-Chrom Q, 80–100 mesh	235	^{63}Ni ECD	Griseofulvin or medazepam	Diethyl ether	

Berlin *et al.*[20] (1972)	6-ft. × 3-mm glass, 3% OV-17 on 60–80 mesh Gas-Chrom Q	240	^{63}Ni ECD	Griseofulvin	Benzene	Used for bioavailability studies on diazepam. Intact drug
Zingales[7] (1973)	120-cm × 2-mm I.D. glass, 2% OV-17 on Chromosorb G, 80–100 mesh	235	^{63}Ni ECD	—	Toluene–heptane–isoamyl alcohol. Acid clean-up for low concentrations	Intact diazepam and metabolites
Baird *et al.*[29] (1973)	3-ft. × $\frac{1}{8}$-in. glass, 3% OV-225 on Gas-Chrom Q, 80–100 mesh	235	^{63}Ni ECD	Prazepam	Diethyl ether with acid clean-up	Suitable for medazepam diazepam and metabolites intact
Mallach *et al.*[30] (1973)	150-cm × 3-mm I.D. glass, 3% OV-25 on Chromosorb W AW DMCS, 80–100 mesh	210–240 (10/min)	Thermionic (NFID)	MACB	Diethyl ether, acid clean-up	Improved sensitivity to medazepam and desmethylmedazepam. Temperature programme
Howard *et al.*[21] (1974)	1-ft. × 4-mm I.D. glass, 3% OV-225 on Gas-Chrom Q, 60–80 mesh	235	^{63}Ni ECD	Prazepam	Ethyl benzoate	Rapid extraction method for toxicology, unsuitable for low concentrations
Knowles and Ruelius[37] (1972)	2-ft. × 2-mm stainless steel, 2% XE-60 on Chromosorb W, 80–100 mesh	240	^{63}Ni ECD	—	Diethyl ether and acid clean-up	Method for oxazepam. Chromatography of ACB
Vessman *et al.*[49] (1972)	6% OV-17	235	Tritium ECD	Lorazepam	Methylene chloride and acid clean-up	Hydrolysis to benzophenones
Belvedere *et al.*[50] (1972)	2-m × 4-mm I.D. glass, 3% OV-17 on Chromosorb Q, 100–120 mesh	260	^{63}Ni ECD	Diazepam	Diethyl ether	Method for temazepam using TMS derivative

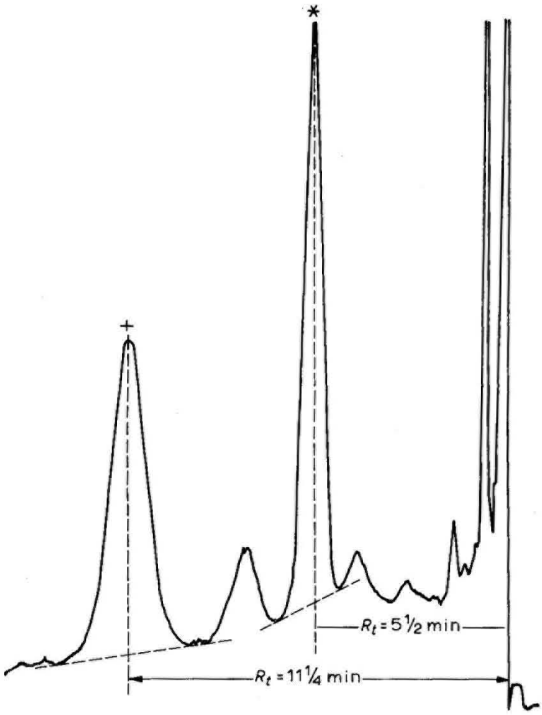


Fig. 3. Chromatogram of diazepam and its N-demethylated metabolite in blood determined as the MACB and ACB compounds, respectively. (Reproduced from ref. 8 with permission of the publishers.)

adsorbents. Although good reproducibility was obtained, internal standards were not used in this early work.

In 1968, Marcucci *et al.*[13] reported the chromatography of the intact benzodiazepines using an OV-1 stationary phase. Flame ionization detection was used in most of the work, with electron capture detection to improve sensitivity in several experiments. The compound 2-N-benzylamino-5-chlorobenzophenone (BACB) was used as an internal standard for the gas chromatography, eluting after the benzodiazepines. Interfering peaks from co-extracted material were not encountered in work with rat blood samples. This work was later presented in greater detail[14]. The method was modified by Van der Kleijn *et al.*[15] to incorporate an OV-17 stationary phase. BACB was again used as the internal standard. The higher temperatures required to elute the benzodiazepines intact necessitated the use of the more stable silicone stationary phases, and high-temperature electron capture detectors incorporating a ^{63}Ni source.

De Silva and Puglisi[6] used an OV-17 phase and a ^{63}Ni electron capture detector for analysis of medazepam, diazepam and their metabolites (Fig. 4). The method incorporated a clean-up procedure to remove lipids and other endogenous co-extracted material. In the diazepam assay, medazepam was used as an internal standard, being taken through the entire extraction and clean-up procedure. Ether extraction was again used, giving recoveries of 86 $\pm$ 6.0% for diazepam and 94 $\pm$ 6.0% for its

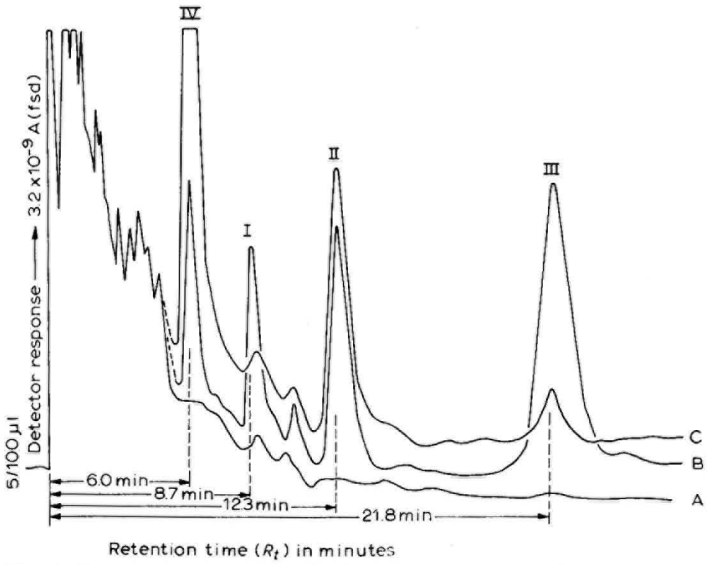


Fig. 4. Gas chromatograms of diethyl ether extracts of (A) patient control urine, (B) control urine containing added authentic standards, and (C) patient urine (0–24 h) post-medication with diazepam; 3% OV-17 stationary phase. I, Diazepam; II, N-desmethyldiazepam; III, 3-hydroxydiazepam; IV, oxazepam. (Reproduced from ref. 6 with permission of the publishers.)

desmethyl metabolite. In urine assays, 80–85% recoveries were reported for oxazepam and 3-hydroxydiazepam. Sensitivity was in the range 0.01–0.04 μg/ml in blood using a 1-ml sample, and could be improved by increasing the sample size.

De Silva and Puglisi[6] discussed a number of factors relevant to gas chromatography with electron capture detection. Concentration of hydrochloric acid used in the back-extraction of ether is critical to benzodiazepine stability. The hydroxy derivatives require the use of 6 *N* hydrochloric acid for quantitative back-extraction, this being verified by TLC studies. In contrast to the earlier work of Marcucci *et al.*[13], OV-1 was not found to be a suitable phase, as resolution of the various compounds from a biological extract was incomplete, resulting in overlapping peaks. Under the conditions used in this assay, hydroxydiazepam had an inconveniently long retention time of 22 min, and a relatively low detector response. The situation could be improved by formation of the trimethylsilyl derivative using hexamethyldisilazane/trimethylchlorosilane reagent. A shortened retention time (12.8 min) was obtained with an approximately 10-fold increase in sensitivity. Overlap with the desmethyldiazepam peak was overcome by a differential extraction technique.

The use of a benzodiazepine internal standard, which could be taken through the entire assay, represented a useful advance. However, the use of medazepam, as reported by De Silva and Puglisi, suffers from some disadvantages. The compound is susceptible to decomposition when stored in solution, has a short retention time, giving possible overlap with impurity peaks, and also a lower electron capture response than diazepam and its metabolites. In addition, back-extraction into hydrochloric acid more concentrated than 2 *N* causes partial conversion of medazepam into a quinoxaline.

There has been widespread use of methods based on those of Marcucci *et al.* and De Silva and Puglisi. Modifications have included minor changes in the chromatography and extraction procedures[16,17], and the use of different internal standards[18].

The method of De Silva and Puglisi is fairly long, because of the clean-up procedure involved. Several groups have recently published methods for the determination of diazepam which involve the use of more rapid extraction procedures. These methods have produced useful results, but in most cases have been used to monitor diazepam levels after overdosage or chronic administration. Quantitation of very low levels of diazepam and its metabolites using electron capture detection will require an acid clean-up stage, as interference due to co-extracted endogenous compounds becomes significant at low levels.

Foster and Frings[19] published a rapid method in which plasma was extracted with chloroform and an aliquot injected on to the chromatograph. Using a flame ionization detector (FID), the method was suitable for toxicological work with high levels of the drug. Chromatography of the metabolites was not reported. Berlin *et al.*[20] used a benzene extraction without clean-up in the determination of bioavailability of diazepam in various formulations. The internal standard was griseofulvin and was added to the sample extract immediately prior to chromatography. Diazepam and its desmethyl metabolite were determined in plasma at steady-state concentrations. Detection limits of 30 and 40 ng/ml, respectively, were quoted using electron capture detection.

Zingales[7] has studied steady-state levels of diazepam and metabolites in plasma, erythrocytes and urine using electron capture GC with an OV-17 phase. The extraction solvent consisted of toluene–heptane–isoamyl alcohol (80:20:1.6). The main purpose of the alcohol was to prevent adsorption of the benzodiazepines on to glass. This extraction mixture was claimed to give cleaner chromatograms than those produced following extraction with ether or chloroform. The method also included a selective extraction procedure whereby each compound could be isolated for further characterization Extraction experiments showed that the toluene–heptane ratio was critical for quantitative extraction of the hydroxy metabolites (Fig. 5). Zingales[7] also gave data for variation of partition ratios of the benzodiazepines as a function of buffer pH and of acid concentration. It was shown that the optimum buffer pH for extraction was between 8.5 and 10, and that 6 *N* hydrochloric acid was required to completely remove the hydroxy metabolites from the extraction solvent. This last result is similar to that obtained by De Silva and Puglisi. Extraction without clean-up was used for routine clinical and toxicological work with acid clean-up being reserved for determinations of very low levels of diazepam and its metabolites. An internal standard was not used in this work.

A rapid extraction method for toxicological work has recently been developed by Howard *et al.*[21]. This followed a procedure for amphetamines described by Ramsey and Campbell[22], but used ethyl benzoate instead of chloroform to permit use of direct injection of the extraction solvent and electron capture detection. Satisfactory results were obtained for diazepam and all its metabolites, with detection limits of 0.02–0.1 μg/ml. The method was not considered suitable for determination of very low levels of the compounds.

Separation of diazepam and its metabolites by high-pressure liquid chro-

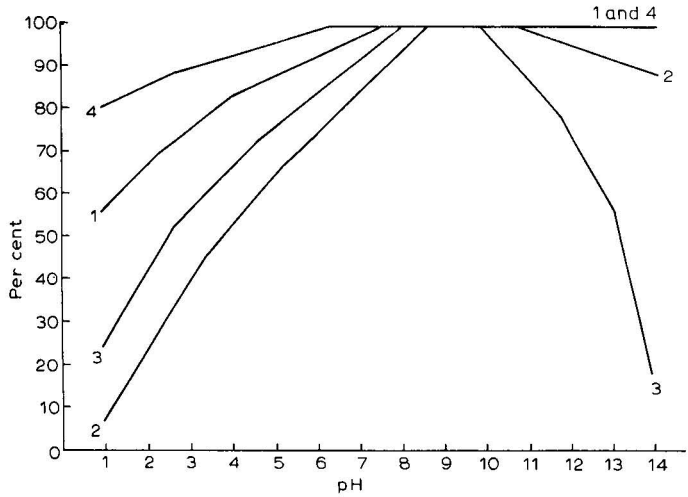


Fig. 5. Partition characteristics of diazepam (1), desmethyldiazepam (2), oxazepam (3) and 3-hydroxydiazepam (4) between toluene–*n*-heptane–isoamyl alcohol and various buffer solutions. (Reproduced from ref. 7 with permission of the publishers.)

matography (HPLC) was reported by Scott and Bommer[23]. Liquid–solid chromatography was carried out using Durapak (OPN), 36–75 μm, in a 100 cm $\times$ 1 mm column with hexane–isopropanol (95:5) as a solvent at a flow-rate of 1.0 ml/min. A UV monitor set at 254 nm was used as detector. Complete resolution of diazepam and its three major metabolites was achieved at a sensitivity of about 2 μg per compound.

Schwartz and co-workers[24,25] used TLC to follow the metabolism of diazepam labelled with ^{3}H in the 5-phenyl ring following administration to dogs and humans. Bands were detected by fluorescence quenching under UV light, and the bands scraped off for spectroscopic characterization. This approach has been adopted for other benzodiazepines during development of the drugs. In the analysis of extracts of biological materials, De Silva *et al.*[9] used a two-dimensional development on silica gel plates, compounds being rendered visible by immersion of the plate in iodine vapour. Ruelius *et al.*[26] also used TLC to separate diazepam from its metabolic products. Jommi *et al.*[27,28] studied diazepam metabolism in rabbits using TLC methods and column chromatography with magnesium silicate and alumina. Further details of the TLC separation of diazepam and its metabolites are given in Table 3.

(b) Medazepam

Medazepam differs from diazepam in having no carbonyl group at C_2. Its main metabolites are shown in Fig. 2, and include diazepam and its biotransformation products. Chromatographic methods for the determination of medazepam are therefore linked to those described for diazepam.

The method of De Silva and Puglisi[6] was the first gas chromatographic assay for medazepam and metabolites in biological samples, and has already been referred to in connection with the assay of diazepam. No internal standard was used. The electron capture detector does not respond well to either medazepam or its desmethyl

TABLE 3

TYPICAL TLC SYSTEMS FOR BENZODIAZEPINES

Compound	*Reference*	*Solvent system*	*Detection*	*Use*
Diazepam	Schwartz *et al.*[24]	(1) Heptane–chloroform–ethanol (10:10:1) (2) Heptane–chloroform–acetic acid–ethanol (5:5:1:0.3) Or isopropanol–concentrated ammonia (20:1)	Iodine vapour and scintillation counting	Metabolic studies using ^{3}H-labelled material Blood level distribution studies
	De Silva *et al.*[9]	Chloroform–acetone (90:10)	UV	
	Beckstead and Smith[117]	Chloroform–toluene–methanol (10:9:1)	UV, modified Dragendorff, or chlorine–toluidine	Separation from intermediates and impurities
Medazepam	Schwartz and Carbone[31]	As for diazepam TLC[24] Also heptane–ethyl acetate–ethanol–conc. ammonia (5:5:1:0.3)	UV and scintillation counting	Metabolic studies on ^{14}C-labelled material
	Lauffer and Schmid[36]	Cyclohexane–diethylamine–benzene (80:15:5)	UV, fluorimetry after treatment with phosphoric acid	Plasma level analysis
Oxazepam	Sisenwine *et al.*[39]	(1) Chloroform–ethanol–acetone (8:1:1) (2) Ethyl acetate–ethanol–ammonia (5:5:1)	UV, Bratton–Marshall	Metabolic studies in animals and man
	Steidinger and Schmid[41]	Toluene–tetrabutylamine–methanol (8:1:1) Or ethyl acetate–dichloromethane (2:5)	Fluorimetry	Metabolic studies in man
	Beckstead and Smith[117]	Toluene–nitromethane–methanol (11:8:1)	UV, Dragendorff	Separation from intermediates and impurities

Chlordiazepoxide	Schwartz and Postma[53]	(1) Chloroform–ethanol (9:1) (2) Ethyl acetate–ethanol (95:5)	UV, scintillation counting	Metabolic studies in rat using ^{14}C-labelled drug
	Beckstead and Smith[117]	Chloroform–methanol (10:1)	UV, modified Dragendorff, chlorine–*o*-toluidine	Separation of chlordiazepoxide hydrochloride from impurities and intermediates
Bromazepam	De Silva and Kaplan[70]	Heptane–chloroform–ethanol (10:10:1) Or ethyl acetate	UV, scintillation counting, GLC	Blood level analysis
	Sawada[71]	Chloroform–acetone (9:1)	Bratton–Marshall, Dragendorff	Metabolic studies in animals
Lorazepam	Schillings *et al.*[82]	Chloroform–ethanol–acetone (8:1:1) Or ethyl acetate–ethanol–ammonia (5:5:1)	UV, Bratton-Marshall	Metabolic studies
Nitrazepam	Rieder[88]	Ethyl acetate–*n*-propranol–diethylamine (70:30:1) Toluene–acetone–ammonia (50:50:1)	UV, Folin's reagent, Bratton–Marshall	Metabolic studies and forensic analysis
	Beckstead and Smith[117]	Chloroform–methanol (10:1) Or chloroform–toluene–methanol (10:9:1)	UV, Bratton–Marshall, chlorine–*o*-toluidine	Separation from impurities and intermediates
Flurazepam	Schwartz and Postma[105]	Ethyl acetate–ethanol–ammonia (95:5:0.5) Benzene–ethyl acetate–ethanol–ammonia (80:20:10:0.2)	UV, scintillation counting	Metabolic studies
Clonazepam	De Silva and Puglisi[104]	*n*-Propanol–benzene–conc. ammonia (80:20:1)	Pulse polarography after UV	Analysis of major urinary metabolites
	Eschenhof[100]	(1) Toluene–acetone–ammonia (50:50:1) (2) Benzene–*n*-propanol–ammonia (80:20:1)	UV, Bratton–Marshall, scintillation counting	Metabolic studies

metabolite (Ro 5-2925), although both compounds are eluted intact from the chromatographic column. The loss of the carbonyl function at position 2 evidently significantly decreases the electron capturing ability of the benzodiazepine system. Following 30-mg doses of the drugs to humans, De Silva and Puglisi were unable to detect desmethylmedazepam in the blood after a single dose (detection limits for this compound and medazepam being about 0.05 μg/ml), while diazepam and the major blood metabolite desmethyldiazepam were readily detected. An alternative procedure for electron capture gas chromatography has been reported by Baird *et al.*[29], in which a more polar silicone phase (OV-225) was used to increase the resolution between diazepam and desmethyldiazepam. This made it possible to use the benzodiazepine prazepam as an internal standard for the assay, this compound eluting between diazepam and desmethyldiazepam (Fig. 6). As in the previous method, sensitivity to medazepam and desmethyldiazepam was low, and the latter could not be detected in the blood following a single therapeutic dose of medazepam (10 mg, orally). The method also has application in the analysis of diazepam, but has the disadvantage that 3-hydroxydiazepam has a high retention volume unless derivatized. In both electron capture gas chromatographic methods, acid clean-up of the extracts is con-

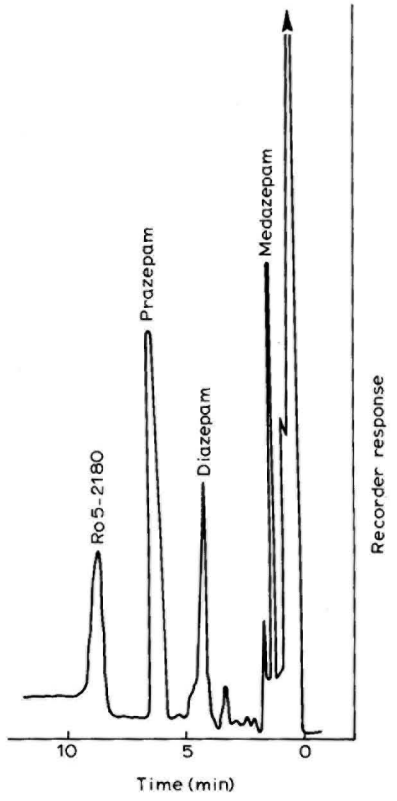


Fig. 6. Gas chromatogram of medazepam and its major metabolites after extraction from plasma. Prazepam internal standard, OV-225 stationary phase. (Reproduced from ref. 29 with permission of the publishers.)

sidered essential, as medazepam's relatively low response and proximity to the solvent front make it necessary to eliminate as far as possible endogenous compounds which would co-chromatograph.

Some of the disadvantages inherent in electron capture chromatography of medazepam have been overcome by Mallach *et al.*[30] by use of a nitrogen-selective (thermionic) detector. Using a 3% OV-25 stationary phase, medazepam and its major benzodiazepine metabolites were resolved by use of a temperature programme from 210° to 240° at 10°/min. The benzophenone MACB was used as an internal standard for quantitative chromatography. Silylation of 3-hydroxydiazepam by the method of De Silva and Puglisi[6] shortened its retention time and again increased its sensitivity. An ether extraction was used with an acid clean-up using hydrochloric acid of two concentrations to take account of the acid instability of medazepam and the difficulty of extraction of the hydroxy metabolites. The method was used successfully in the analysis of medazepam and metabolites in human serum and urine following therapeutic dosage, although large sample volumes of sera (4 ml) were used. Compared with electron capture detection, the thermionic detector provided a significant improvement in sensitivity for medazepam and desmethylmedazepam. Use of a temperature programme greatly improved the resolution of metabolites, although it was not stated whether the system used could also resolve the major urinary metabolite 2-amino-3-benzoyl-5-chlorophenol reported by Schwartz and Carbone[31].

Improved sensitivity to medazepam and desmethylmedazepam has also been achieved by Hailey *et al.*[32] using a Coulson detector. No co-extracted material was detectable in the analysis of spiked rat blood samples, and using an OV-17 stationary phase, temperature programming was successfully used to increase resolution. The OV-225 phase used in previous work[21] was unsuitable for temperature programming with this detector owing to the high bleed-off of nitrogenous material.

The use of temperature programming seems especially suitable in the analysis of medazepam and its metabolites by GLC. Even with isothermal operation, however, resolution of medazepam from the solvent front can be improved by use of a non-silicone stationary phase, and Howard[33] used the polyimide phase Poly I-110 to increase the relative retention time of medazepam from that obtained on OV-225.

TLC separation of [^{14}C]medazepam and its metabolites has been used by Rieder and Rentsch[34] and by Schwartz and Carbone[31] in studies on the metabolism of the compound in several species. Two-dimensional TLC on silica gel plates was carried out in similar fashion to that described by Schwartz and co-workers for diazepam metabolites.

Besserer *et al.*[35] reported the determination of medazepam by TLC following ether extraction of serum. Lauffer and Schmid[36] used spectrofluorimetry to quantitate medazepam following separation by TLC. The substances were located by fluorescence quenching under UV light (254 nm), and also after spraying the plate with various reagents. Fluorescence spectra were measured on plate (using a scanner) and in solution. Strong fluorescence was achieved by pre-treating the plate with 0.1 *N* hydrochloric acid and then spraying it with orthophosphoric acid. The method was used to measure medazepam in plasma and gastric juice and was used to monitor the blood levels in humans following a therapeutic dose.

(c) Oxazepam

The diazepam metabolite oxazepam has been marketed as a tranquilliser for some years. It differs from diazepam and non-hydroxylated benzodiazepines in that a substantial proportion of the drug in the bloodstream is present as the glucuronide[37].

As with other benzodiazepines, metabolic studies were carried out with the aid of the ^{14}C-labelled compound, and Walkenstein *et al.*[38] used ascending paper chromatography to separate urinary metabolites from various animal species. Separation of oxazepam and its glucuronide was achieved, and it was noted that rat urine contained several other metabolites, although they were not identified. The metabolism was further investigated by Sisenwine *et al.*[39] using a two-dimensional TLC system on silica gel F-254 plates. As well as the use of analytical TLC, metabolites were identified by NMR and mass spectrometry after separations on a preparative scale. Metabolites identified included benzodiazepines with phenolic or methoxy substituents on the 5-phenyl ring, and also a number of open-chain compounds. It was suggested that oxazepam might be in equilibrium with the tautomeric opened ring.

Weist[40] has described a TLC system for oxazepam which can be used as a preparation for subsequent quantitative determination by fluorimetry. The drug was detected on plate after heating with 70% perchloric acid, and the method was used in the analysis of oxazepam in various body fluids. Steidinger and Schmid[41] used a thin-layer scanner to measure urinary oxazepam. Urine samples were incubated with β-glucuronidase, extracted with dichloromethane, and the dried residue was chromatographed on silica gel. Fluorimetric assay was carried out on plate following treatment with trichloroacetic acid or by eluting oxazepam from the plate and determining it separately by spectrofluorimetry.

Kamm and Kelm[42] used TLC of the hydrolysis product (ACB) followed by diazo coupling with an azo dye and spectrophotometry to measure oxazepam blood levels. Sunjic *et al.*[43] used TLC and column chromatography to separate the diastereoisomers of oxazepam-camphanic acid esters. Hydrolysis of the esters resulted in a racemate.

Gas chromatography has also been widely used to determine oxazepam. When diazepam and its metabolites are gas chromatographed, it is observed that oxazepam is eluted first, despite being the most polar compound. This chromatographic behaviour is due to thermal decomposition. Oxazepam rapidly loses a molecule of water on column, forming 6-chloro-4-phenylquinazoline-2-carboxaldehyde (Fig. 7). This compound is more volatile than diazepam and its other two major metabolites, which are eluted intact. The decomposition process was investigated by Sadee and Van der Kleijn[44] and by Forgione *et al.*[45] using GLC–MS and direct mass spectrometry. The mechanism of dehydration was demonstrated by use of ^{18}O- or ^{2}H-labelled oxazepam. Conversion of oxazepam into its decomposition product was almost 100%. Medazepam, desmethylmedazepam, diazepam and desmethyldiazepam were eluted intact, but 3-hydroxydiazepam gave a smaller response than expected with total ion current detection, suggesting partial decomposition. As the decomposition of oxazepam is rapid and almost quantitative, quantitation without hydrolysis to ACB is still possible. Preparation of the trimethylsilyl derivative of oxazepam gives a peak with a retention time similar to that of the decomposition product[6]. More

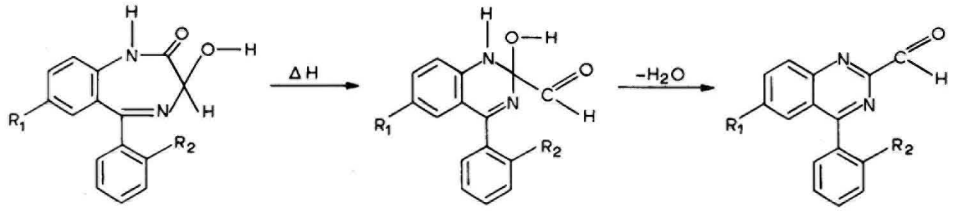


Fig. 7. Thermal decomposition of N-desalkyl-3-hydroxybenzodiazepin-2-ones.

recently, Frigerio *et al.*[46] have shown that similar decompositions occur for lorazepam and 3-hydroxynitrazepam, GC–MS again being used. Similar results have been obtained with 3-hydroxybromazepam and 3-hydroxydesalkylflurazepam[47] and would appear to be general for the N-desalkyl-3-hydroxybenzodiazepines.

Knowles and Ruelius[37] reported an electron capture gas chromatographic method for determining the drug in biological fluids which has been used to measure blood levels following therapeutic dosage. The method was a modification of that of De Silva *et al.*[9]. Oxazepam was extracted from phosphate-buffered serum with ether and back-extracted into 12 *N* sulphuric acid. The acid layer was washed with ether and then heated to 100° for 1 h to hydrolyze oxazepam to the benzophenone ACB, which was chromatographed on XE-60 stationary phase. An external standard technique was used to check the instrument performance. The oxazepam glucuronide in blood was determined in a similar manner after incubation with β-glucuronidase. This hydrolysis method was preferred to chromatography of the intact compound as cleaner chromatograms and higher sensitivity were obtained. A sensitivity limit of 20 ng/ml was quoted, compared with 50 ng/ml for the intact compound mentioned by Marcucci *et al.*[48]. Use of the hydrolysis method implied, as usual, some loss of specificity, but this is less of a problem in the case of oxazepam compared with most other marketed benzodiazepines, as metabolites other than the oxazepam glucuronide are present only in very small amounts.

Vessman *et al.*[49] have also published a GLC method for oxazepam and its glucuronide based on electron capture detection of ACB. Free oxazepam was extracted from serum buffered at pH 7.4 with methylene chloride containing lorazepam as internal standard. The benzodiazepines were back-extracted into sulphuric acid and hydrolyzed before re-extraction and chromatography. Quantitative determinations down to 1 ng/ml were performed. This work included a discussion on optimum extraction and hydrolysis conditions.

(d) Temazepam

3-Hydroxydiazepam (temazepam) has been used in a number of clinical studies, and a gas chromatographic method was reported by Belvedere *et al.*[50]. A similar procedure to that mentioned by De Silva and Puglisi[6] was used, temazepam being silylated at the hydroxy group before chromatography on 3% OV-17. Diazepam was used as an internal standard. Like De Silva and Puglisi, the authors reported an increase in sensitivity to electron capture detection following silylation.

B. Chlordiazepoxide

Chlordiazepoxide was the first of the benzodiazepines to be marketed, and its metabolic pathways (Fig. 8) were established by use of TLC separation in conjunction with the radioactively labelled drug. Thus, Koechlin and co-workers[51,52] used TLC and paper chromatography with [2-^{14}C]chlordiazepoxide to study the metabolism of the compound in man and dog, and established that demoxepam was a plasma metabolite in both species. Quantitation was achieved using a chromatogram scanner, by scintillation counting after elution of the spots, or by hydrolysis followed by a Bratton–Marshall reaction. Ethyl acetate-extractable urinary metabolites were isolated in milligram amounts using a silica gel column. A subsequent radio-TLC study by Schwartz and Postma[53] showed the presence of a third metabolite, desmethylchlordiazepoxide, in man. A further metabolic study in the rat[54] used two-dimensional TLC with reference compounds and high-resolution mass spectrometry to establish the identity of the urinary metabolites. Pribilla[55] developed TLC systems for chlordiazepoxide and its metabolites and applied them to the analysis of urine, blood and tissue samples. A large number of solvent systems and detection methods were tested and compared. The most reliable method involved hydrolysis to the benzophenone ACB followed, if required, by diazotisation and coupling.

Urinary chlordiazepoxide metabolites were also studied by Kimmel and Walkenstein[56] using TLC and paper chromatography of ^{14}C-labelled material

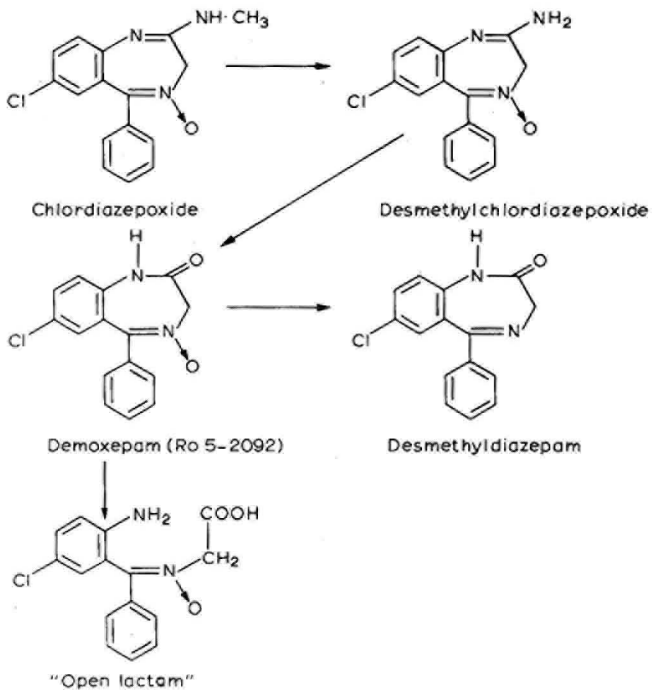


Fig. 8. Metabolic pathways for chlordiazepoxide.

followed by autoradiography and scintillation counting. Kaplan *et al.*[57], in a pharmacokinetic study in the dog, used TLC for urine samples to eliminate interfering fluorescence. Miachon and Revol[58] used TLC and paper chromatography in their study on chlordiazepoxide metabolism in rabbits, with fluorimetric and Bratton–Marshall detection.

Schwartz *et al.*[59] have used two-dimensional TLC with NMR and mass spectroscopy in studying the metabolites of [2-^{14}C]demoxepam in the dog. Several metabolites were identified in the dog with phenolic groups in either benzenoid ring. In man[60], demoxepam was the major urinary metabolite with small amounts of oxazepam and the 9- and 4-hydroxy-N-oxides. Desmethyldiazepam was the only faecal metabolite identified. Spots were located under UV light and then removed and quantitated by scintillation counting.

Brooks *et al.*[61] have reported a toxicological assay for chlordiazepoxide and its desmethyl and lactam metabolites using pulse polarography to determine the compounds after separation on silica gel. The sensitivity of the assay is about 0.5 μg/ml.

The identification and determination of desmethyldiazepam in human plasma following chronic administration of chlordiazepoxide has been reported by Dixon *et al.*[62] using a combination of TLC and electron capture GC. The results obtained gave good agreement with those found using a radioimmunoassay technique that was specific for desmethyldiazepam in the presence of chlordiazepoxide and its other metabolites.

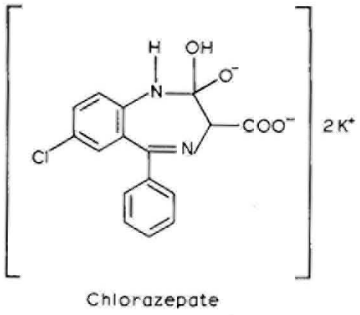
Analytical HPLC of chlordiazepoxide was reported by Scott and Bommer[23] in their study on the separation of the benzodiazepines from biological material. Using a UV detector, a sensitivity in the microgram range was reported.

The first gas chromatographic method for chlordiazepoxide was described by De Silva[63], and involved ether extraction of the compound from blood, followed by hydrolysis to ACB prior to chromatography. This method obviously did not distinguish between the drug and its metabolites. Martin and Street[64] chromatographed chlordiazepoxide without prior hydrolysis using heat-treated (350°) SE-30 on silanized Chromosorb W and a stainless-steel column at a temperature of 245°. However, under these conditions two peaks were obtained, indicating possible decomposition on the column. Possibly as a result of the difficulties with GLC analysis of chlordiazepoxide, the most widely used method for the drug and its metabolites in body fluids in recent years has involved spectrofluorimetry[53,65]. However, in 1971, Zingales[66] reported the successful gas chromatography of intact chlordiazepoxide using electron capture detection. In this method, the drug was extracted with *n*-heptane containing 1.5% isoamyl alcohol from plasma buffered to pH 9. A recovery of about 90% was achieved. At high concentrations of the drug, a portion of the extraction liquid was injected directly on to the chromatographic column. With low plasma levels, the drug was back-extracted into 0.1 *N* hydrochloric acid before re-extraction and concentration. Higher recoveries (97–100%) were obtained at pH 7.4, but the higher pH value was chosen because the parent drug is selectively extracted under these conditions. Analyses were carried out using a 4 ft. × $\frac{1}{4}$ in. O.D. column packed with 2% OV-17 on 80–100 mesh Chromosorb W, at an oven temperature of 275°. This different chromatographic behaviour presumably reflected the use of improved packing materials and more careful column treatment.

Although nitrones such as demoxepam can be chromatographed by GLC, the results are not satisfactory from the point of view of quantitation, as compounds of this type were found by Sadee and Van der Kleijn[44] to partially decompose on the column by loss of oxygen from the N_4 position.

C. Chlorazepate

Chlorazepate incorporates a carboxylic acid function in the diazepine ring (Fig. 9). In acid solution, it is quickly converted into desmethyldiazepam[67]. The main metabolic products are desmethyldiazepam and oxazepam. Gros and Raveux[68] reported the TLC of chlorazepate on silica gel G using as solvent *n*-butanol–methanol–formamide (70:15:5). At 4°, R_F values of 0.20 and 0.80 were obtained for the drug and desmethyldiazepam, respectively, and the spots were made visible by fluorescence quenching.



Chlorazepate

Fig. 9. Chlorazepate.

Analysis of chlorazepate and its metabolites in blood and urine was described by Viala *et al.*[69]. The metabolites are ether extracted from blood buffered to pH 9, and processed for GLC analysis as described above under *Diazepam*. Chlorazepate is retained in the aqueous phase, which is then adjusted to pH 3 and heated at 40° for 5 min. Under these conditions, chlorazepate is converted to desmethyldiazepam, which is ether extracted after readjustment of the aqueous phase to pH 9, and chromatographed in the usual way.

D. Bromazepam

Bromazepam has recently been marketed in Europe as a hypnotic, and differs from other benzodiazepines in having a 7-bromo substituent and a 5-pyridyl rather than a 5-benzyl ring (Fig. 10). TLC and electron capture gas chromatography of this compound have been described by De Silva and Kaplan[70]. TLC was carried out on silica gel using either ethyl acetate or *n*-heptane–chloroform–ethanol as solvents, and fluorescence quenching was employed for rendering the spots visible. Sawada and co-workers[71,72] used TLC to study urinary metabolites of bromazepam, with standard detection techniques and additional spectroscopic characterization.

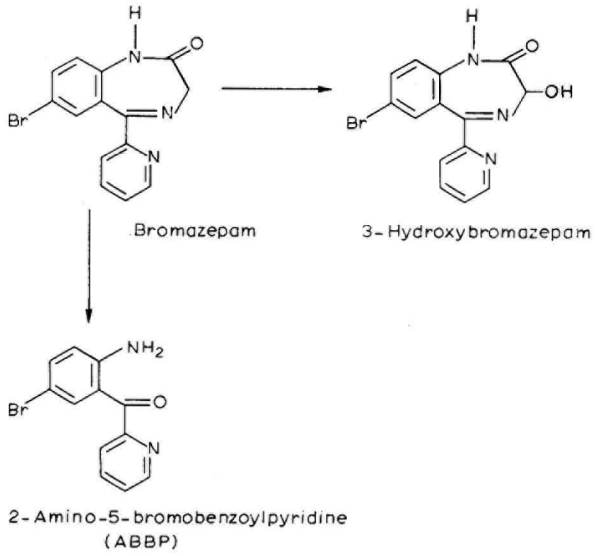


Fig. 10. Bromazepam and metabolites.

Preliminary GLC studies by De Silva and Kaplan[70] showed that intact bromazepam was thermally unstable at the temperatures required for elution. Analysis was therefore carried out by hydrolysis with 6 *N* sulphuric acid to give 2-amino-5-bromobenzoylpyridine (ABBP), which was chromatographed on a 2-ft. stainless-steel column containing 2% Carbowax 20M–TPA. No internal standard was used, and a minimum detectable level of 5.0 ng was reported. The overall recovery from blood was 61 ± 3%. This low recovery was attributed in part to further hydrolysis of ABBP to *p*-bromoaniline and nicotinic acid.

Greaves[73] has successfully chromatographed bromazepam intact at the microgram level, using flame ionization detection and an OV-17 stationary phase. Treatment of the compound with BSTFA apparently produced a suitable silyl derivative, giving a single sharp peak. Successful electron capture gas chromatography of intact bromazepam at the nanogram level has recently been reported by De Silva *et al.*[74].

E. Oxazolobenzodiazepines

The 5,4-oxazolobenzodiazepine derivatives oxazolam and cloxazolam (Fig. 11) have undergone clinical studies. Their metabolism in the rat has been studied by TLC of the ^{14}C-labelled material in conjunction with IR, UV, NMR and mass spectroscopic data[75,76]. Oxazolam metabolites included desmethyldiazepam, oxazepam and several benzophenones.

In one of the few papers on HPLC of the benzodiazepines to be published so far, Weber[77] described the analysis of the related compound ketazolam. This compound cannot be analyzed using GLC as it is immediately pyrolyzed to give diazepam. This is unacceptable as diazepam is used in the synthesis of ketazolam, and is also a metabolic product. The two compounds were separated on a 1-m Corasil II column

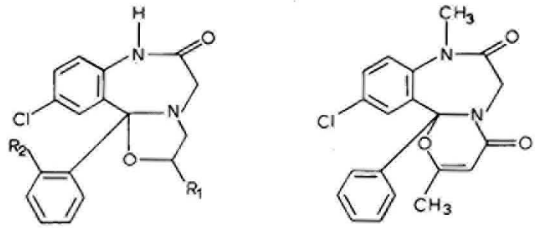


Fig. 11. Formulae of oxazolobenzodiazepines.

(Fig. 12) using a mixture of tetrahydrofuran and diisopropyl ether (15:85) as eluent. Detection was by UV absorbance at 254 nm, with a full-scale reading equivalent to 0.02 A. Sensitivity limits (amounts injected on column) were 5 ng for diazepam and 30 ng for ketazolam. Repeated sampling of the test solutions was used to check on the rate of conversion of ketazolam into diazepam in the tetrahydrofuran–diisopropyl

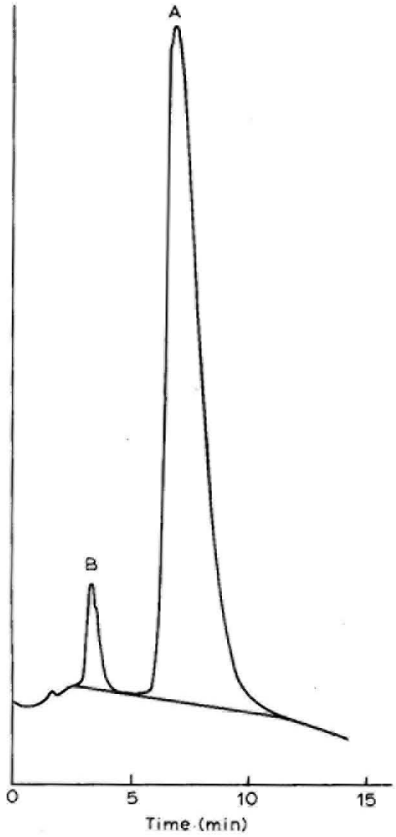


Fig. 12. HPLC separation of ketazolam and diazepam. (Reproduced from ref. 77 with permission of the publishers.)

ether solvent. Extension of the method to oxazepam and nitrazepam gave limits of 15 and 40 ng, respectively. These sensitivities represented approximately a 1000-fold improvement from the levels chromatographed by Scott and Bommer[23] and probably give a good indication of the results to be expected using current commercially available UV detectors.

F. Prazepam

Prazepam (Fig. 13) has been used in clinical trials, although it is not yet marketed. Its metabolites include a number of other benzodiazepines already considered. Prazepam metabolites in dog, rat and mouse have been investigated by Di Carlo and co-workers[78–80] using the 2-^{14}C-labelled compound and TLC. Spots were detected by use of a radiochromatogram scanner, Bratton–Marshall reaction with benzophenone hydrolysis products, or reaction with iodine. Quantitative results were obtained by scintillometry. Prazepam metabolism in man was studied by similar methods[81]. Co-chromatography with known standards was used with a radiochromatogram scanner.

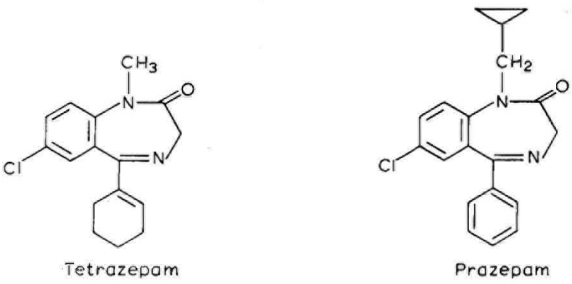


Fig. 13. Tetrazepam and prazepam.

Gas chromatography of prazepam and its metabolites following therapeutic dosage has not yet been reported. However, prazepam itself has extraction and chromatographic characteristics very similar to those of diazepam[29], and electron capture gas chromatography of biological extracts containing the drug and its metabolites using the type of approach already described for diazepam and medazepam should present little difficulty.

G. Lorazepam

Lorazepam is a derivative of oxazepam, differing in having an additional chlorine atom substituted at the 2′-position in the 5-phenyl ring (Fig. 14). It is a more potent drug than oxazepam and consequently both doses and body fluid levels are lower. Following therapeutic dosage, urinary metabolites of the drug were studied by Schillings *et al.*[82] using TLC methods in conjunction with mass spectroscopy and infrared spectroscopy. Similar methods to those used for oxazepam were employed, incorporating two-dimensional chromatography on silica gel plates, and similar detection techniques. The major urinary metabolite was again the glucuronide, other

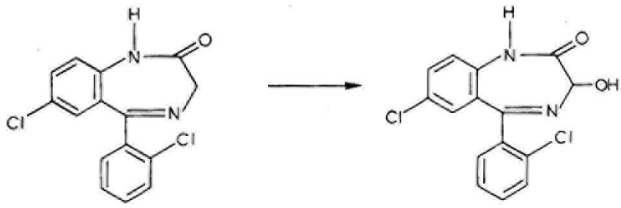


Fig. 14. Ro 5-3027 and lorazepam.

compounds accounting for less than 1% of the total excreted. Schuetz and Schuetz[83] have described a rapid detection method for the drug in urine by acid hydrolysis, ether extraction of the benzophenone (ADCB), and TLC with the spots being made visible with Bratton–Marshall reagent.

Knowles *et al.*[84] developed an electron capture gas chromatographic method similar to that for oxazepam. Serum buffered at pH 7 was ether extracted, and the benzodiazepine then back-extracted into 12 *N* sulphuric acid and hydrolyzed at 100°. The conjugated drug was determined after β-glucuronidase incubation of the extracted aqueous layer. These workers used a 10 ft. $\times$ 2 mm I.D. stainless-steel column packed with 3% OV-17 on Chromosorb W, 100–120 mesh, with a column temperature of 280°. The method was sufficiently sensitive to measure serum and urine concentrations of lorazepam even after 2-mg doses, with a lower level of 0.01 μg/ml.

Marcucci *et al.*[85] have chromatographed lorazepam intact on 3% OV-17–Gas-Chrom Q (100–120 mesh) with a sensitivity limit of 1 ng/ml of blood. A 78% extraction recovery was reported, and BACB was added as internal standard prior to chromatography. Gas chromatography of intact lorazepam has also been discussed by De Silva *et al.*[86] in connection with the benzodiazepine Ro 5-3027 ("2′-chlorodiazepam"), which has lorazepam as its main metabolite. In this system, hydrolysis to the benzophenone is undesirable because of loss of specificity. Using a method similar to that for medazepam and diazepam[6], it was found that the apparent recovery of lorazepam from blood was only 40 $\pm$ 6.0%, which was attributed to adsorption phenomena on column, especially with older columns. Reproducible trimethylsilyl derivatives could not be prepared. It was also noted that sensitivity to the electron capture detector (ECD) was enhanced on addition of the compounds to blood, compared with the response obtained from standard solutions. This suggested the formation of adsorption complexes or chemical derivatives in the presence of impurities. Similar, but smaller, enhancement could be obtained from addition to 6 *N* hydrochloric acid. These authors also consider the possibility that on-column conversion to the quinazoline carboxaldehyde may not be quantitative in the presence of impurities.

H. 7-Nitrobenzodiazepines

Several compounds which have been used clinically have higher potencies than that of diazepam, and are therefore often given in lower doses and produce lower blood and tissue levels. These include the compounds containing a 7-nitro group and/or an

additional halogen substituent in the 2′-position. The low concentrations obviously place increased demands on the chromatographic methods, both from the point of view of absolute sensitivity and exclusion of interfering substances. Adsorption, oxidation and photodecomposition become more significant processes at these low levels. Because of these difficulties, it is evident that methods evolved for analysis of the drug in quality control or in overdose situations may not be suitable for clinical work or for pharmacokinetics.

Metabolic pathways for the 7-nitrobenzodiazepines are indicated in Fig. 15. In addition to the routes shown, N-demethylation has also to be considered in the case of flunitrazepam and nimetazepam. The major blood metabolites of nitrazepam and clonazepam have low pharmacological activity[87], and it may often be appropriate in clinical pharmacology to analyse only the parent drug.

The gas chromatography of 7-nitrobenzodiazepines is summarized in Table 4.

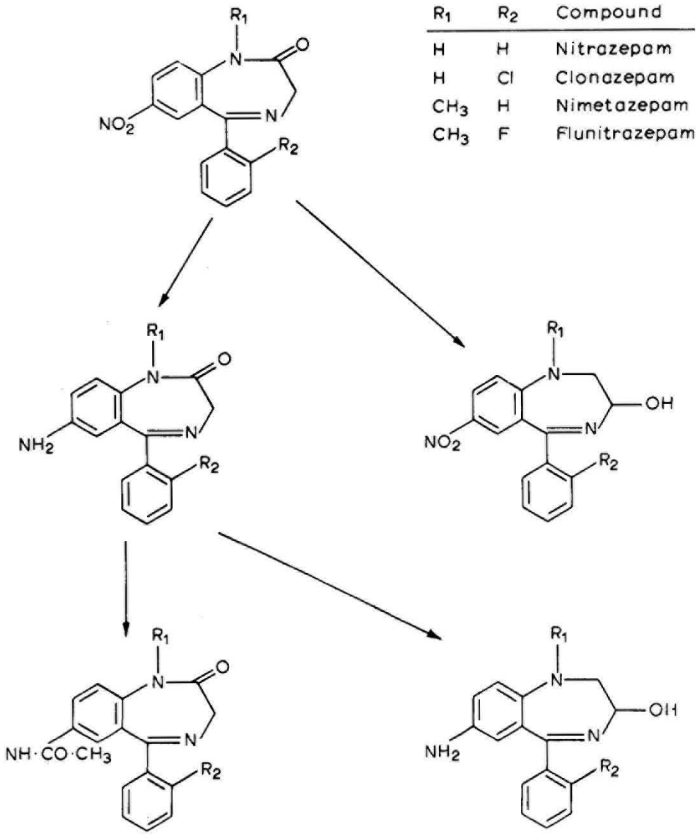


Fig. 15. Metabolic pathways for the 7-nitrobenzodiazepines.

(a) Nitrazepam

Nitrazepam is widely used as a sleep inducer and anticonvulsant. As with other benzodiazepines, the metabolism of nitrazepam was studied using TLC sepa-

TABLE 4

GAS CHROMATOGRAPHY OF THE 7-NITROBENZODIAZEPINES

Benzodiazepine	*Workers*	*Column*	*Detector*	*Internal standard*	*Extraction and work-up*	*Comments*
Nitrazepam	Lafargue *et al.*[130] (1970)	2-m, 3% OV-17 on Gas-Chrom Q, 100–120 mesh $T = 250°$	FID	—	—	Chromatography of intact nitrazepam and two metabolites, and hydrolysis products. Microgram amounts injected on column; poor peak shapes
	Viala *et al.*[69] (1971)	1.20-m glass, 10% SE-52 on Chromosorb W, $T = 210–270°$ (programme)	FID	—	Diethyl ether or 2:1 dichloromethane–ethyl acetate. Acid hydrolysis to benzophenones	Method for toxicological analysis
	Beharrell *et al.*[95] (1972)	1.8-m × 4-mm I.D. glass, 3% OV-17 on Gas-Chrom Q, 60–80 mesh, $T = 235°$	^{63}Ni ECD	Clonazepam (ANCB)	Diethyl ether, acid clean-up and hydrolysis to benzophenones	Suitable for plasma levels after therapeutic dose. Metabolites not detected
	Ehrsson and Tilly[96] (1973)	0.9-m × 2-mm I.D. glass, 5% OV-17 on Gas-Chrom Q, 80–100 mesh, $T = 250°$	^{63}Ni ECD	Griseofulvin	Benzene, then methylation	Suitable for plasma levels after therapeutic dose. Metabolites not detected
Clonazepam	Næstoft *et al.*[102] (1973)	3-ft. × 4-mm I.D. glass, 1% OV-17, on Celite CQ, 100–120 mesh	^{63}Ni ECD	Desmethyldiazepam	Toluene–isoamyl alcohol, acid clean-up	Plasma levels after therapeutic dose. Possible interference from metabolite. Intact drug chromatographed
	Næstoft *et al.*[103] (1974)		^{63}Ni ECD	(1) Desmethylflunitrazepam (2) Acetylated Ro 10-3384	Ethyl acetate, acid clean-up and differential extraction	Suitable for chromatography of drug and two major metabolites after therapeutic dosage
	De Silva and Puglisi[104] (1974)	4-ft. × 4-mm I.D. glass, 3% OV-17 on 60–80 mesh Gas-Chrom Q, $T = 230°$	^{63}Ni ECD	Flunitrazepam (MANFB)	Diethyl ether extraction, acid clean-up and hydrolysis to benzophenones	Used for blood level determinations following therapeutic dosage. Metabolites not detected
Flunitrazepam	De Silva and Puglisi[104] (1974)	3-ft. × 4-mm I.D. glass, 3% OV-225 on 60–80 mesh Gas-Chrom Q, $T = 230°$	^{63}Ni ECD	Clonazepam (ANCB)	As above	Blood level determinations of drugs and desmethyl metabolite

ration of the ^{14}C-labelled compound. The spots were rendered visible by autoradiography and the eluted radioactive material was measured by scintillometry. In 1965, Rieder[88] published TLC data on nitrazepam and its two major blood metabolites. The spots were made visible with either Folin's reagent (grey-blue spot) or Bratton–Marshall reagent (blue-red), which gave more sensitive detection. Pribilla[89] also used TLC to study the excretion of nitrazepam in humans.

Oelschlager and co-workers[90,92,93] separated nitrazepam and its metabolites on magnesium silicate plates and determined the compounds by d.c. polarography. Areas of adsorbent containing the compounds were scraped off and shaken with dimethylformamide (to reduce adsorption) before analysis.

Scott and Bommer[23] and Weber[77] chromatographed nitrazepam with HPLC systems using UV detection.

Gas chromatography of nitrazepam was reported by Matsuda[94], who obtained usable chromatograms for the hydrolysis product 2-amino-5-nitrobenzophenone (ANB), with flame ionization detection. Nitrazepam gave a broad peak with pronounced tailing. High concentrations were chromatographed, and the method was not suitable for the determination of the drug following therapeutic doses. Marcucci *et al.*[13] included nitrazepam in their work on diazepam chromatography, but once again high levels were chromatographed and no work was carried out on detecting the drug in biological fluids. Hydrolysis to ANB was also described by Viala *et al.*[69], who followed the work of Rieder[88] in developing toxicological analytical methods for the drug and its metabolites using TLC with a Bratton–Marshall reaction. Gas chromatography of ANB was carried out on 10% SE-52 on Chromosorb W using flame ionization detection.

In 1972, Beharrell *et al.*[95] reported a method for determining nitrazepam in biological samples using an OV-17 stationary phase and electron capture detection, which was capable of quantitation of the drug following therapeutic doses. Clonazepam was used as an internal standard. Reproducible results could not be obtained with the intact molecules below about 1.0 μg/ml, and hydrolysis with hydrochloric acid to the benzophenones (ANB and ANCB) was therefore used. The results are shown in Fig. 16. All glassware was silanized to minimise adsorption processes. The method was not suitable for the amino and acetamido metabolites, which are weakly electron capturing and hydrolyze to a benzophenone (DAB), which also has a low affinity for electrons. Ehrsson and Tilly[96] reported an electron capture GC method for nitrazepam which eliminated the need for acid hydrolysis. This was made possible by methylation of the N_1 position using iodomethane–tetrabutylammonium hydrogen sulphate after benzene extraction of the drug. Glassware was silanized before use. The major metabolites did not interfere in the chromatography, being well separated from methylnitrazepam and the internal standard, and also giving a much lower ECD response. The method was suitable for quantitation of nitrazepam in the range 5–100 ng/ml (Fig. 17).

It seems probable that the GLC of nitrazepam will develop in similar fashion to that of diazepam. With improved column technology, it should be possible to chromatograph the compound intact even at the low levels (1–20 ng/ml) expected after therapeutic doses, and thereby eliminate the necessity for hydrolysis or methylation. Increased ECD sensitivity to the 7-amino metabolites can be achieved by reaction with pentafluoropropionic anhydride to give the fluoroacyl derivative[97]. In

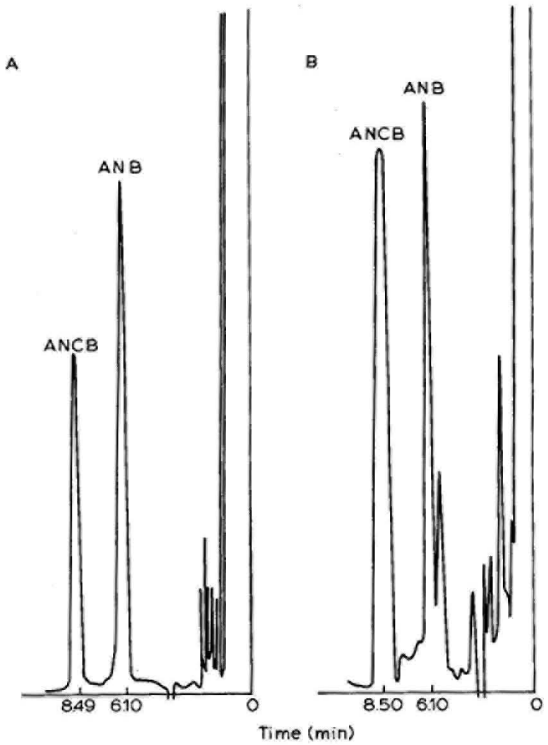


Fig. 16. Chromatograms of ANB–ANCB mixtures. (A) standard solution, ANB concentration 0.04 μg/ml; (B) plasma sample, ANB concentration 0.025 μg/ml. 3% OV-17 stationary phase. (Reproduced from ref. 95 with permission of the publishers.)

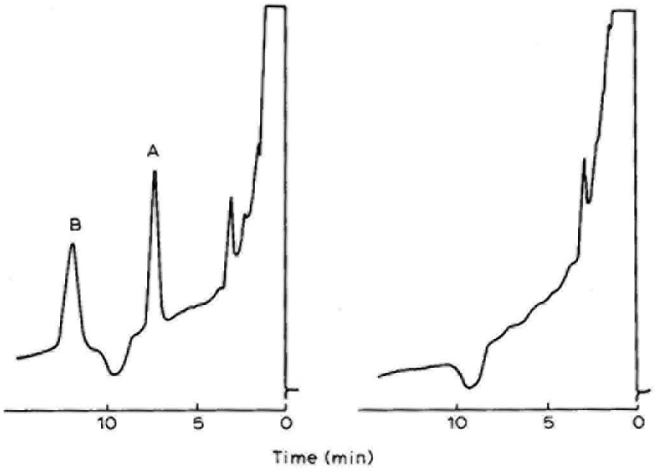


Fig. 17. (a) Gas chromatogram from human plasma containing 40 ng/ml of nitrazepam, A = methylnitrazepam, B = griseofulvin; (b) chromatogram from human blank plasma (no addition of internal standard). 5% OV-17 stationary phase. Electrometer setting: 4×10^{-10}. (Reproduced from ref. 96 with permission of the publishers.)

most of the work carried out so far on the determination of nitrazepam metabolites in body fluids, the fluorimetric method described by Rieder[98] has been used.

It is of interest to compare the different electron capturing ability of nitrazepam and its metabolites with the situation for medazepam mentioned previously. In the case of nitrazepam, removal of the electronegative substituent at position 7 (by reduction) greatly reduces the ECD response, although the diazepin-2-one structure is unchanged. Medazepam has a relatively weak electron-capturing ability, despite the presence of a halogen atom at C_7. Introduction of the carbonyl group (giving diazepam) greatly increases the response.

(b) Clonazepam

Clonazepam has been used clinically as an anticonvulsant. As with nitrazepam, body tissue and fluid levels are very low, making demands on the analytical method. TLC methods were used to elucidate the metabolism of the compound[99,100].

A method for the intact drug in human serum following administration of 2- and 4-mg doses was described by Næstoft *et al.*[101,102]. These workers extracted the drug from plasma with toluene containing isoamyl alcohol, and following a clean-up procedure with hydrochloric acid and heptane, used electron capture gas chromatography with desmethyldiazepam as an internal standard (Fig. 18). Interference

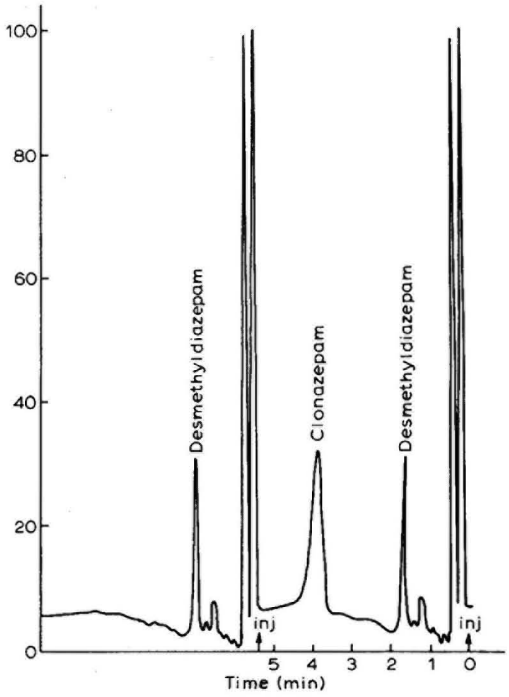


Fig. 18. Chromatograms of an extract from a plasma sample from a patient not receiving clonazepam, blind with added internal standard (left), and an extract from a plasma sample from one patient receiving clonazepam (right). 1% OV-17 stationary phase. (Reproduced from ref. 102 with permission of the publishers.)

by the 7-amino metabolite was considered a possibility. Subsequently, Næstoft and Larsen[103] improved the method to make possible the separate quantitation of clonazepam and both major metabolites. In the modified assay, clonazepam is first separated from its metabolites by differential extraction. It is then chromatographed on OV-17 with desmethylflunitrazepam as internal standard. The two metabolites are chromatographed separately on the same column with 7-acetamido-2′-chloro-1-methyl-1,4-benzodiazepin-2-one as internal standard. Detection limits were 3–5 ng/ml.

In contrast to this work, De Silva *et al.*[104] found that electron capture detection of intact clonazepam gave unsatisfactory results, broad and poorly defined peaks being obtained on both OV-1 and OV-17 phases. Hydrolysis to ANCB gave a well defined peak, and was capable of being used to assay clonazepam at the nanogram level. Possible interference due to the presence of the 3-hydroxy metabolite (which also hydrolyzes to ANCB) was eliminated because of the different partition characteristics of this compound during the extraction procedure. Flunitrazepam (Ro 5-4200) was used as an internal standard, this compound hydrolyzing to the benzophenone MANFB. Using an OV-17 stationary phase, the detection limit for blood samples was between 0.5 and 1.0 ng/ml, using a 2-ml sample. For optimum electron capture detection, it is important that trace amounts of water are not introduced on injection of the sample. Extracted residues were therefore vacuum dried and dissolved in acetone–hexane which had been dried over anhydrous sodium sulphate. Quantitative extraction was achieved with ether from borate buffered plasma. The method was not suitable for the 7-amino metabolite of clonazepam, as hydrolysis gave an unexpected product with a very poor electron capture response, more than 200 ng being required to produce a measurable peak.

This method for clonazepam was associated with a similar one for flunitrazepam (Ro 5-4200) and its desmethyl metabolite, clonazepam in this second assay being used as internal standard. Apparent recoveries of Ro 5-4200 from blood were greater than 100%, suggesting that complex formation with blood-extracted impurities occurred. Flunitrazepam and its desmethyl metabolite are eluted intact on a GLC column with good peak shape, but a benzophenone method is required as both are unstable in acid, which is used in the essential clean-up procedure. In the flunitrazepam assay, OV-225 was used as stationary phase rather than OV-17, giving a better separation of the three benzophenones MANFB, ANFB and ANCB.

The hydrolysis medium used in both assays was a mixture of 4 *N* hydrochloric acid and 4 *N* sulphuric acid (1:1), as hydrochloric acid alone sometimes resulted in chlorination of ANCB. This was especially true with old hydrochloric acid, and it was suggested that an increase in free oxygen concentration gave rise to free or activated chlorine in solution. Trace metal-catalyzed chlorination was another possibility. In the case of MANFB and ANFB, it is essential to carry out the neutralization step following the hydrolysis carefully at low temperature (ice cooling) as the 2′-fluorobenzophenones are readily converted into the acridones on warming in a basic medium. These observations may have relevance to other benzodiazepine assays which involve hydrolysis to the benzophenones.

In addition to the GLC assay, De Silva and Puglisi[104] analyzed major urinary metabolites of clonazepam and flunitrazepam by TLC linked to pulse polarography. A one-dimensional separation on silica gel was carried out and the compounds were eluted with two 5-ml portions of methanol and dissolved in 0.1 *N* hydrochloric acid

for polarographic analysis. Using the wave due to reduction of the azomethine moiety (−0.6 V *versus* SCE), the detection limit was 0.5–0.75 μg with a 5-ml sample. This is considered to be suitable for the analysis of urinary metabolites following chronic administration of the drugs.

I. Flurazepam

Flurazepam differs from other benzodiazepines currently marketed in having a large substituent (diethylaminoethyl) at N_1 (Fig. 19). The most important metabolic processes involve degradation of this side-chain, 3-hydroxylation playing a relatively minor role[105]. The major blood metabolites are the hydroxyethyl and desalkyl compounds, flurazepam itself being rapidly eliminated from the circulation. The levels following a therapeutic dose are very low (1–15 ng/ml), and gas chromatographic techniques have been only partially successful in the analysis of these compounds. De Silva and Puglisi[106] developed an electron capture gas chromatographic method based on the benzophenones obtained by acid hydrolysis. Although this method was useful for toxicological work, it was not suitable for use with humans who had

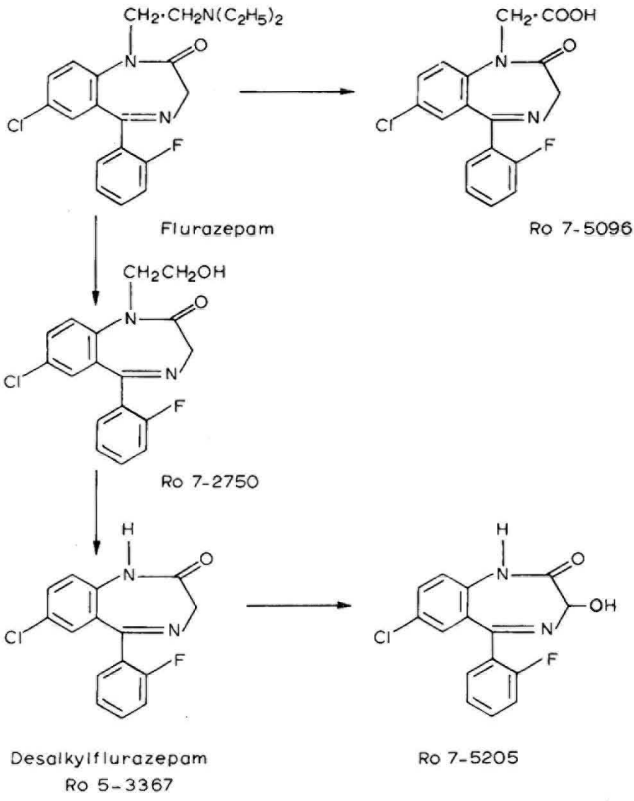


Fig. 19. Metabolic pathways of flurazepam.

received therapeutic doses of flurazepam, as the limit of sensitivity was of the order of 0.02 μg/ml of plasma.

A more sensitive assay was therefore developed in which flurazepam and its metabolites were determined fluorimetrically following TLC separation[107]. The compounds were selectively extracted into ether, back-extracted into 4 *N* hydrochloric acid, and hydrolyzed to the benzophenones. The hydrolysate was made alkaline, and extracted with ether. The residue obtained after evaporation was dissolved in dimethylformamide, potassium carbonate added, and the mixture heated at 110° for 2 h to convert the benzophenones to the highly fluorescent 9-acridones. Following re-extraction, these were separated by TLC on silica gel plates using chloroform–acetone (85:15) as solvent. The areas on the TLC plate corresponding to the acridones was scraped off, the acridones eluted with methanol–0.1 *N* hydrochloric acid (80:20), and their fluorescence measured with a spectrofluorimeter.

In the analysis of urinary metabolites, it is sometimes possible to determine the major metabolites by TLC of the benzophenones followed by spectrophotometry, but the levels of the compounds are often too low, and it is then necessary to resort to the more sensitive fluorimetric assay.

Schwartz and co-workers[105,108] used combined TLC and mass spectrometry to identify urinary metabolites of flurazepam. Separation was carried out on fluorescent silica gel and a solvent consisting of ethyl acetate–ethanol–12 *N* ammonia (90:10:0.3) for the first dimension. A variety of other solvents was used in the isolation of each metabolite. Detection was achieved by spraying with modified Dragendorff reagent. The metabolism of flurazepam in man was followed by similar chromatography of ^{14}C-labelled material. The spots were scraped off the plate after development and counted by scintillometry.

The fluorimetric assay does not distinguish between N-desalkylflurazepam and the desalkyl-3-hydroxy metabolite, as both give rise to the same acridone. However, Kaplan *et al.*[109] were able to show that the 3-hydroxy compound was not present in the blood of subjects who had received 30 mg flurazepam orally for 2 weeks. An electron capture GC procedure was used that completely resolved the two compounds, and this confirmed that the N-desalkyl compound was the major blood metabolite.

3. CHROMATOGRAPHIC ANALYSIS OF THE BENZODIAZEPINES IN THE BULK DRUGS AND IN FORMULATIONS

Many chromatographic methods have been described for the analysis of the drugs, their decomposition products and their intermediates for quality control purposes[110–130].

Beckstead and Smith[117] described in considerable detail a TLC scheme for the detection of impurities in benzodiazepines. These authors also included a brief review of the earlier literature on the TLC of chlordiazepoxide, diazepam, nitrazepam and oxazepam. TLC methods for these four drugs and their impurities were then described. A number of solvent systems and spray reagents were investigated to separate the benzodiazepines from intermediates in their synthesis and their hydrolysis or decomposition products. The variation in polarity between the compounds ruled out a single TLC system, and a series of systems was developed for quality control handling of each benzodiazepine (Table 3). Silica gel containing a fluorescent indicator was found

to be the most useful adsorbent, and fluorescence quenching was a good general method of detection, detection limits being better than 1 μg. Of the spray reagents, chlorine–*o*-toluidine and modified Dragendorff–cerium(IV) sulphate were the most sensitive and generally applicable. Bratton–Marshall reagent gave high sensitivity for compounds with a primary amino group. Iodoplatinate reagent was considered unsatisfactory because of the wide variation in sensitivity with related compounds. The scheme was used to analyse a number of commercially available benzodiazepine formulations and bulk drug samples.

Arizan *et al.*[119] used TLC and polarography to study various stages in the synthesis of diazepam. Dragendorff reagent was used to render the spots visible, polarography being used for quantitation. Bich *et al.*[120] used a TLC–spectrophotometer system to measure individual active agents in drug formulations, including chlordiazepoxide, eliminating the need to elute the compounds from the plate. The sensitivity was in the microgram range. Mayer *et al.*[124] used TLC techniques to study the hydrolysis and decomposition of diazepam and nitrazepam during storage and showed that the rate of decomposition/hydrolysis depended on the moisture content and the presence of auxiliary ingredients with a large surface area. TLC methods for chlordiazepoxide and diazepam have been summarized by MacDonald *et al.*[126].

Haefelfinger[129] used the reagent 2,5-dimethoxytetrahydrofuran–*p*-dimethylaminobenzaldehyde to locate primary amines on TLC plates. It was noted that both nitrazepam and diazepam gave positive results, unlike medazepam or chlordiazepoxide. The anomaly was explained by suggesting that with the two reacting compounds, the diazepine ring opened, giving a primary amino group. Analytical methods for determination of stability of drug parenterals have been discussed by Johnson and Venturella[122], who included the GLC and TLC analysis of diazepam.

Fricke[125] has described a semi-automated GLC procedure for a variety of drugs, including chlordiazepoxide and diazepam, using Dexsil 300 as the stationary phase. Lafargue *et al.*[130] described a GLC study of a number of benzodiazepines and some of their metabolites and hydrolysis products. Sample sizes of 2 or 4 μg were injected on a column of OV-17 on 100–120 mesh Gas-Chrom Q using flame ionization detection. The compounds studied included chlorazepate, which was impossible to chromatograph, and tetrazepam, which was eluted between oxazepam and diazepam. The nitrazepam metabolites Ro 5-3072 and Ro 5-3308 were eluted intact but with poor peak shape and pronounced tailing. Desmethylchlordiazepoxide gave three peaks, indicating thermal instability. Retention data were included for all compounds studied.

4. CHROMATOGRAPHIC ANALYSIS OF THE BENZODIAZEPINES IN CLINICAL TOXICOLOGY

A great number of general screening procedures for drugs in urine and blood, ncluding one or more benzodiazepines, have been published[131–162]. In many cases, metabolites were not taken into consideration in these methods, and the benzophenone hydrolysis products were chromatographed rather than the intact drugs. A comprehensive bibliography of these screening methods has not been included in this review, but the literature cited here is thought to cover the most important work in this field. There are also a number of publications which deal with the benzodiazepines

alone[69,73,91,163–175], and which contain methods to distinguish between the different compounds and their metabolites. The various TLC systems used in benzodiazepine analysis have tended to use rather similar methods of development and detection, and the paper by Beckstead and Smith[117] referred to previously provides a useful summary of the earlier work.

The cheapest and most widely used screening procedures have used TLC systems, usually in conjunction with spray reagents. Fluorescence quenching has also been popular. In addition, many of the benzodiazepines rearrange to highly fluorescent products on treatment with concentrated acids, giving rise to very sensitive detection methods (Table 5).

TABLE 5

FLUORESCENCE DATA FOR BENZODIAZEPINES

From Lafargue *et al.*[173]

Compound	*Acid*	$\lambda_{max.}$ *excitation (nm)*	$\lambda_{max.}$ *emission (nm)*
Chlordiazepoxide	H_2SO_4	310	530
Diazepam	H_2SO_4	295	490
Chlorazepate	H_2SO_4	388	508
Medazepam	H_2SO_4	345	485
Oxazepam	H_3PO_4	360	475
Nitrazepam	$HClO_4$	300	465
Tetrazepam	H_3PO_4	398	492

Bellemonte[167] used UV and IR spectrophotometry in conjunction with TLC to determine diazepam and its metabolites in urine, following hydrolysis to the benzophenones. Oelschlager mentioned the TLC separation of chlordiazepoxide, diazepam and nitrazepam and their metabolites in an early review of the compounds[112], and reported d.c. polarography as being suitable for quantitation. Zingales[137] used an ether extraction followed by TLC using five chromatographic systems and several colour reactions to identify a number of psychotropic drugs, including diazepam and chlordiazepoxide. Weist and Schmid[142] described a rapid TLC method for benzodiazepines and other drugs on micro-plates, which was used for the detection of the compounds in cases of poisoning.

De Silva and D'Arconte[169] mentioned the use of TLC linked to fluorimetry in the forensic analysis of chlordiazepoxide, and on-plate spectrofluorimetry was used by Lauffer and Schmid[141] in their separation scheme for 60 drugs, including chlordiazepoxide. Alha and Lukari[170] used diazotisation and coupling of 7-aminonitrazepam and ACB in the TLC of forensic samples. For quantitative work, the spots were eluted and measured spectrophotometrically. Sawada and Shinohara[172] used a TLC system to identify nitrazepam and its metabolites in *post mortem* samples. Using a series of solvents and spray reagents, the method was also applied to chlordiazepoxide, oxazepam and diazepam. Lafargue *et al.*[173] gave details of a rapid method for benzodiazepines in toxicological cases, using fluorescent alumina plates to separate the hydrolysis products. Metland *et al.*[157] used XAD-2 ion-exchange resin to remove the drugs from urine before elution and TLC with a number of spray reagents. Schuetz *et al.*[175] have described the two-dimensional TLC of five benzodiazepines, the compounds being converted into the benzophenones on plate by

spraying with hydrochloric acid, and then being reacted with Bratton–Marshall reagent. Berry and Grove[161] identified benzodiazepines in urine by heating the samples with 1 *N* hydrochloric acid in an autoclave at 15 p.s.i. for 15 min, and then extracting with light petroleum, chromatographing the benzophenones on silica gel, and coupling with N-naphthylethylenediamine to give an azo dye. The method is sensitive, being capable of detecting urine levels after therapeutic dosage, but is limited in specificity as oxazepam, chlordiazepoxide and desmethyldiazepam give the same hydrolysis product (ACB). Medazepam and diazepam are not detectable by this method. Nitrazepam hydrolyzes to the benzophenone ANB, which is separable from ACB. In addition, the 7-acetamido metabolite of nitrazepam gives a blue fluorescence under UV light after chromatography with methanol–12 *N* ammonia (100:1.5).

Systems based on GLC using flame ionization detection, or GLC in combination with TLC, have also been widely used. Vignoli and Cano[164] chromatographed ACB, MACB and ANB on an SE-52 stationary phase using FID, reporting detection limits of 60 ng for diazepam and nitrazepam and 90 ng for chlordiazepoxide. Finkle *et al.*[152] included chlordiazepoxide, diazepam, medazepam, oxazepam, flurazepam and nitrazepam in their screening procedure based on direct solvent extraction and a GLC system utilizing four columns and three stationary phases. A sensitivity limit of 2 μg/ml was reported. Flurazepam was chromatographed at 250°, and the other three compounds at 200° on an SE-30 stationary phase. The primary purpose of this work was to provide retention data for a large number of drugs. Proelss and Lohmann[151] included chlordiazepoxide, diazepam and oxazepam in a screening method for 40 sedatives and tranquillisers. Benzodiazepines were ether extracted from buffered serum at pH 8. A number of stationary phases were evaluated, and the best results were obtained with 3% OV-17, this being the only phase which resolved all clinically important phenothiazines, dibenzazepines and benzodiazepines. Phenothiazine was used as an internal standard.

Viala *et al.*[69] described a combined GLC and TLC procedure for the toxicological analysis of benzodiazepines in blood and urine. Account was taken of the major metabolites, and the compounds chlordiazepoxide, diazepam, medazepam, nitrazepam, chlorazepate and oxazepam were included. Most of the work made use of hydrolysis to the benzophenones in order to obtain increased sensitivity by GLC. As a number of benzodiazepines give ACB as a hydrolysis product, GLC of the intact compounds was also used for identification purposes. Flame ionization detection was used in most cases, although electron capture detection was mentioned for the analysis of ACB, MACB, medazepam and desmethyldiazepam. A TLC system on silica gel plates was also described. Interferences by overlapping benzodiazepine spots could be eliminated using different detection methods. Interference by other drugs was not considered.

Sine *et al.*[155] used GLC with flame ionization detection following chloroform extraction of serum buffered at pH 7.4. A caffeine internal standard was used, and the column contained 3.8% SE-30 on Chrom W. Gardner-Thorpe *et al.* published TLC[149] and GLC[156] systems for determining anticonvulsants in blood and included chlordiazepoxide, diazepam, nitrazepam and oxazepam. TLC on silica gel gave detection levels of less than 1 μg for the benzodiazepines when viewed under UV light. In the GLC work, several columns were evaluated and retention data reported. No

suitable system for nitrazepam was obtained. The other benzodiazepines were separated on 3% SE-30 at 250° and detected using flame ionization.

Law *et al.*[154] described a GC–MS system with computer storage for low-resolution mass spectra of 58 drugs including chlordiazepoxide and diazepam. The method was based on comparison between *m/e* values of the five strongest peaks in the mass spectra and both drugs were detected in serum from subjects following drug overdose. Finkle and Taylor[153] have also published details of a GC–MS reference system for drug identification which included chlordiazepoxide, diazepam and medazepam. Data were obtained by chromatography on 2.5% SE-30 followed by detection with a quadrupole mass spectrometer. Mass spectral data were numerically coded and compared with stored reference data for final identification. Simple chloroform extractions were used, and the mass spectra references were obtained by injection of 50–100 ng of drug on the column. Unknown peaks were matched by a.m.u. value of the mass spectral base peak and by the most intense peak in every 14 a.m.u. from 43 to 463 *m/e*.

Greaves[73] has reported the quantitative determination of medazepam, diazepam and nitrazepam in whole blood by flame ionization GLC. The method was suitable for toxicological analysis of the compounds following overdosage or chronic therapeutic administration in the case of medazepam and diazepam. OV-1 and OV-17 stationary phases were used. Blood samples were ether extracted on a vortex mixer and, after acid clean-up, the combined extracts were passed through anhydrous sodium sulphate before evaporation. Nitrazepam was reacted with BSTFA to form a TMS derivative which was eluted on OV-17 with the same retention time as diazepam. It was shown that a number of other commonly used drugs did not interfere in the assay.

5. FUTURE TRENDS

It is very probable that a number of other benzodiazepines will before long be available for clinical use.

A number of the new compounds that can be expected to be marketed in the future will be administered in low doses because of increased potency compared with earlier drugs of this type. Flunitrazepam has already been referred to, and the 6-phenyl-4H-5-triazolo[4,3-*a*] compounds have also shown high activity and low toxicity[176]. Analytical methods for these compounds will need to be highly sensitive and selective. As with benzodiazepines that are already available clinically, a number of metabolites will be pharmacologically active, and methods for their determination will need to be developed. Consideration of the chemistry of the 1,4-diazepine ring system and of interferences to be expected from other compounds will be important in the development of chromatographic and other analytical methods.

The possibility of complex formation with biological material, as suggested by De Silva and co-workers[86,104], needs fuller investigation. Further work might also be done in developing more selective procedures for extraction of the drugs and metabolites from body fluids. Use of a structurally similar benzodiazepine as an internal standard for both extraction and chromatography is considered highly desirable, especially in the analysis of the drugs at concentrations less than 20 ng/ml.

Prescription of any psychoactive drug implies eventual misuse and overdosage

by a proportion of the population. Overdosage of benzodiazepines is at present very common[177], and the introduction of further compounds of this type will increase the work of the clinical toxicologist. Revised TLC screening methods will be needed, and for some of the more potent drugs, very sensitive spray reagents highly desirable. Conversion to fluorescent derivatives such as acridones will continue to find application. Caille *et al.*[178] have recently characterized the fluophore produced by treatment of ethanolic solutions of oxazepam with phosphonic acid, and shown it to be the trimer of the thermolysis product 6-chloro-2-formyl-4-phenylquinazoline. Some of the benzodiazepines with more complex structures may not be so amenable to gas chromatography as the earlier members of the series, and TLC followed by formation of a suitable fluophore will continue to provide a useful alternative. De Silva *et al.*[179] have described the TLC–fluorimetric assay of an indolyl-1,4-benzodiazepine, making use of conversion to the fluorescent quinolone after treatment with sulphuric acid.

Gas chromatography is likely to continue to be of major importance for benzodiazepine analysis. Electron capture detection will probably retain its place, especially for the more potent drugs, as the most useful method for monitoring blood and urine levels after therapeutic dosage. Introduction of newer designs of electron capture detector, with a wide linear range and possibly better sensitivity, will make the application of this technique easier. For the analysis of benzodiazepines that give higher body fluid and tissue levels, thermionic and conductivity detectors could become far more widely used, being potentially attractive in terms of the simple extraction and clean-up procedures required. GLC–MS has obvious attractions, especially when used for mass fragmentography. With a favourable fragmentation pattern, good sensitivity and very high selectivity can be achieved, and there is the possibility of being able to use simple extraction procedures without further clean-up, even for very low levels of the drug. This could be particularly advantageous in the case of compounds such as flunitrazepam, which decompose in acid.

In addition to GC–MS applications, use of direct mass spectrometric analysis seems a possibility. Boerner *et al.*[180] have recently reported the use of this technique in analyzing drugs in body fluids in acutely poisoned patients. A chemical vapour analysis system with a computer-linked quadrupole mass spectrometer was used. Drugs analyzed included benzodiazepines. This method has the advantage over chromatographic techniques in being very rapid. This could be of use in the management of some overdosage cases where identification of the drug of abuse is important. However, the technique does not have the sensitivity of some chromatographic methods, and involves the use of expensive instrumentation which may not be available to the hospital laboratory. This method could also find application in metabolic studies. Direct mass spectrometry of a number of glucuronides, including that of oxazepam, has been reported by Billets *et al.*[181].

The use of HPLC seems likely to increase, especially with the availability of less expensive apparatus and more sensitive detectors such as fluorimeters. This technique (in its ion-exchange mode) should be particularly suitable for the analysis of urinary metabolites, many of which have so far not been identified. Even with currently available detectors, blood level determinations of a number of benzodiazepines would seem to be feasible.

For the routine determination of marketed benzodiazepines, immunoassay would appear to offer a number of advantages. Peskar and Spector[182] have developed

a radioimmunoassay for diazepam and desmethyldiazepam. A detection limit of about 1 ng was achieved with a linear range of 1–100 ng. A possible disadvantage of such a method is cross-reaction with other benzodiazepines or metabolites. In the case of the antisera used by Peskar and Spector, it was shown that medazepam, desmethylmedazepam, chlordiazepoxide, demoxepam and oxazepam did not bind to the antibody. However, the binding of compounds with a 2-carbonyl function and no 3-hydroxy or 5-N-oxide substituent (*e.g.*, nitrazepam) were not reported.

Polarographic methods should provide a useful alternative to GLC for quantitation of benzodiazepines, especially in overdose situations. The technique has been used by a number of workers. For example, Berry[183] has used polarography in the determination of diazepam and chlordiazepoxide in plasma and urine, and Halvorsen and Jacobsen[184] have measured nitrazepam and metabolites in horse plasma. Limitations of the technique are sensitivity, which is not yet as good as that of the best GLC detectors, and resolution of the drugs and their metabolites. Resolution of electrochemically very similar benzodiazepines generally requires a prior TLC separation[61,94] or a selective extraction technique.

The analysis of benzodiazepines will continue to demand skilled application of modern analytical techniques. Chromatographic methods can be expected to play a major part in the analysis of these drugs.

6. ACKNOWLEDGEMENT

I thank Mrs. D. M. Turner for her patience in typing the manuscript and preparing a number of figures.

7. SUMMARY

A review is presented of chromatographic methods of analysis for the 1,4-benzodiazepine drugs. Particular emphasis is placed on their determination in biological materials, with discussion on the difficulties involved in this type of work. A short section on chromatographic methods in quality control is included, and the review is concluded by a brief discussion of future developments in the field.

NOTE BY EDITOR

After the submission of this manuscript, a review by J. M. Clifford and W. F. Smyth was published [*Analyst (London)*, 99 (1974) 241] on the determination of 1,4-benzodiazepines and their metabolites in body fluids. The two reviews overlap as far as the chromatographic aspects of the analysis of body fluids is concerned.

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APPARATUS

N-529

MODULAR GAS CHROMATOGRAPH PERFORMANCE

Perkin-Elmer's March, 1974 "Chromatography Newsletter" gives a analysis of the analytical performance of the model 3920 series gas chromatographs. The instrument's modular design allows tailoring to individual customer needs.

The newsletter also includes articles on "Electron capture system with expanded analytical capabilities" and "Applications of the new nitrogen detector" used in analyzing biological samples.

N-546

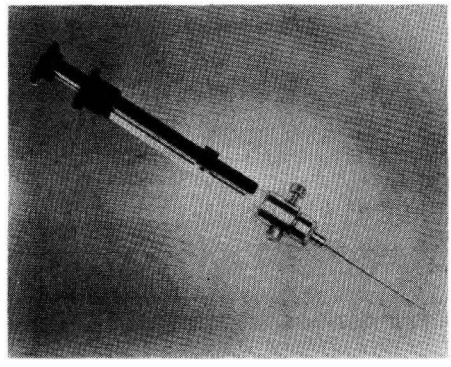
ENVIRONMENT MONITORING

The April 1974 issue of "Short Notes" published by Carlo Erba describes several instruments applied in environmental monitoring. Among them are: the model 420 automatic total organic and inorganic carbon monitor, the model AF 150 centrifugal separator for removal of water and oil from gaseous samples, the series 1700 Sorpty for rapid surface area determination and the series 3500 Porosimeter. Also, an application of the Fractomatic process gas chromatograph in the automatic analysis of the effluents of a naphtha steam-cracking furnace is described.

N-522

HOLDING VALVE FOR MICRO SYRINGES

A valve from Unimetrics locks the sample in the syringe. Valve body and needle are stainless steel with Teflon seals. A threated fitting allows attaching to seven Unimetrics micro syringes with capacities ranging from 50 μl to 10 ml. The sample is drawn into the syringe and held by pushing a slide valve. Valve and syringe combinations are leak-proof to 5 atm. A gas sample can be pressurized prior to injection to produce a complete slug, free of burble or back flow carrier gas.



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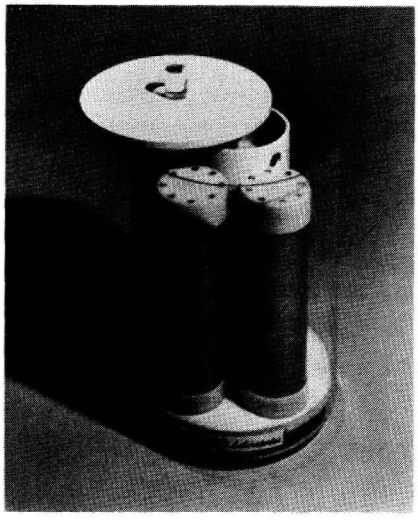
N-527

ELECTROPHORESIS SLAB DESTAINER

Thin electrophoresis gel slabs can be fixed, stained, rinsed and destained easily with the model 222 Slab diffusion destainer from Bio-Rad Labs. Specially designed mesh slab holders protect thin slabs through all the procedures, at the same time exposing the entire gel surface to the solutions. Destaining, therefore, proceeds rapidly and separations can be seen within 30 min. Two types of adsorbent cartridges are available: charcoal for routine stain removal and an optional ion exchange cartridge for working with ampholytes. The model 222 will accomodate two 150 X 150 mm gel slabs at the same time. Optional mesh folders are available for gel slabs up to 150 X 300 mm.



CHEMICALS

N-544

AGAROSE GEL FOR AFFINITY CHROMATOGRAPHY

Affi-Gel 10 from Bio-Rad Labs. allows one-step affinity chromatography with a high-purity agarose gel bead in form with added aliphatic arms 10 Å long, terminated by active carboxy N-hydroxysuccinimide esters. For affinity chromatography, enzyme immobilisation or immunosorption, Affi-Gel 10 is rapid and easy to use.

Packaged one dry gram to an oversize bottle, the ligand containing buffer can be added directly to the Affi-Gel 10 in the bottle, for the coupling reaction. The swelled coupled sorbent is ready for use. No intermediate coupling reactions and no purification steps to rid the materials of excess EDAC. This means less danger of side reactions. Affi-Gel 10 has an aliphatic arm extending wide away from the gel matrix, eliminating steric hindrance and preserving a greater proportion of the free solution binding specificity of coupled ligands.

Immunosorbents prepared with Affi-Gel 10 are more efficient than those prepared by direct cyanogen bromide coupling to agarose gel beads.

N-538

PREPACKED COLUMNS FOR PERMEATION CHROMATOGRAPHY

Redi-Pak™ is a prepacked column for analytical and preparative permeation chromatography utilizing Controlled-Pore Glass. Fourteen pore sizes are available for separating molecular weights from 3,000 to 10^9 and beyond. The columns can be prepared for use in min and operated or stored in any position without bed settling, leakage or drying out. Eluant connections and sample injection are accomplished via hypodermic needles and septums. The column can be autoclaved. Empty Redi-Pak columns are also available for use with any non-swelling, rigid column packing.

N-539

HPLC PACKING MATERIAL AND COLUMNS

Macherey-Nagel has released a 19-page brochure in German on high-pressure liquid chromatography. It contains information on conventional packing materials, like silica, alumina ion-exchangers etc., 5 porous layer bead packings partially with chemically bonded phases Vydac™, filling techniques, micro columns and also a related bibliography.

PROCEDURES

N-542

PHARMACEUTICAL ANALYSES

"Analysis of Pharmaceutical Products", a publication from Waters Ass. describes the total capability in pharmaceutical analyses – analyses of raw materials, final dosage forms, plastic containers, and the determination of drug stability and product uniformity – provided by high-speed liquid chromatography. This one analytical technique can be used to monitor pharmaceutical products at any stage of their development, up to and including final dosage forms.

Using liquid chromatography for quality control analyses is particularly suitable because of rapid analysis times and ease of sample preparation. Neither derivatization nor vaporization is necessary.

N-543

INSTANT TLC

Gelman Technical Bulletin 17R, entitled "Gelman chromatography system" – based on the instant thin-layer procedure, includes background history on thin-layer chromatography and instant thin layer chromatography (ITLC™), detailed ITLC procedures and a current list of 70 references covering the broad uses of ITLC media.

A special section in the Bulletin covers Seprachrom™ techniques. Seprachrom is a miniature thin-layer chromatography system based on a disposable microchromatography chamber utilizing all types of ITLC for such standard applications as drug screening, determination of L/S Ratios, and the quality control of Technetium-99m.

N-545

LIQUID CHROMATOGRAPHY

"Chromatography Notes", describes briefly some of the most recent advances in LC. With its attached reply card, "Chromatography Notes" provides the reader with an easy means of access to detailed information on the subjects of interest to him. Among the topics covered in the two most recent issues are: high-speed gel permeation chromatography, rapid optimization of yield in synthesis reactions, analyzing the preservatives in food products, quantitation in pharmaceutical analyses, sample clarification, a strong cation exchange packing material, high-efficiency reverse phase packing, analysis of phtalate plasticizers, water-compatible GPC packing, purification of 7-hydroxy-1-THC and peptide & protein separations.

The articles are mostly abstracts of detailed publications from Waters Ass. available on request.

CALENDAR OF FORTHCOMING MEETINGS

November 18–22, 1974
Atlantic City, N.J., U.S.A.

Federation of Analytical Chemistry and Spectroscopy Societies, 1st Annual Meeting

Contact:
J.G. Grasselli, Standard Oil Co. (Ohio), 4440 Warrensville Ctr. Rd., Cleveland, Ohio 44128, U.S.A.

March 3–7, 1975
Cleveland, Ohio, U.S.A.

26th Pittsburg Conference on Analytical Chemistry and Applied Spectroscopy

Contact:
P.M. Castle (Program Chairman), 1975 Pittsburgh Conference, Building 401, Room 4A31, Westinghouse Research Labs., Beulah Road, Pittsburgh, Pa. 15235, U.S.A.
(Further details published in Vol. 96, No. 2).

June 1975
Knoxville, Tenn., U.S.A.

28th Annual Summer Symposium on New Horizons in Analytical Spectroscopy

Contact:
J. Winefordner, Dept. of Chemistry, University of Florida, Gainesville, Fla. 32601, U.S.A.

October 6–10, 1975
Indianapolis, Ind., U.S.A.

Federation of Analytical Chemistry and Spectroscopy Societies, 2nd Annual Meeting

Contact:
J.G. Graselli, Standard Oil Co. (Ohio), 4440 Warrensville Ctr. Rd., Cleveland, Ohio 44128, U.S.A.

PUBLICATION SCHEDULE FOR 1974

Journal of Chromatography (incorporating *Chromatographic Reviews*)

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
JOURNAL	88/1 88/2	89/1 89/2	90/1	90/2 91	92/1 92/2	93/1 93/2	94 95/1	95/2 96/1	96/2	97/1 97/2	99 100/1 100/2	
REVIEWS*			98/1						98/2	98/3		

* Volume 98 will consist of *Chromatographic Reviews*. The issues comprising this volume will not be published consecutively, but will appear at various times in the course of the year.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. (a) Original research work not previously published in a generally accessible language in other periodicals (Full-length papers). (b) Review articles. (c) Short communications and Notes. (d) Book reviews; News; Announcements. (e) Bibliography of Paper Chromatography, Thin-Layer Chromatography, Column Chromatography, Gas Chromatography and Electrophoretic Techniques. (f) Chromatographic Data.

Submission of Papers. Three copies of manuscripts in English, French or German should be sent to: Editorial office of the Journal of Chromatography, P.O. Box 681, Amsterdam, The Netherlands. For *Review articles*, an outline of the proposed article should first be forwarded to the Editorial office for preliminary discussion prior to preparation.

Manuscripts. The manuscript should be typed with double spacing on pages of uniform size and should be accompanied by a separate title page. The name and the complete address of the author to whom proofs are to be sent should be given on this page. Authors of papers in French or German are requested to supply an English translation of the title. A short running title of not more than 50 letters (including spaces between the words) is also required for Full-length papers and Review articles. All illustrations, photographs, tables, etc., should be on separate sheets.

Heading. The title of the paper should be concise and informative. The title should be followed by the authors' full names, academic or professional affiliations, and addresses.

Summary. Full-length papers and Review articles should have a summary of 50–100 words. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes will be published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Particular attention should be paid to the size of the lettering to ensure that it does not become unreadable after reduction. Sharp, glossy photographs are required to obtain good halftones. Each illustration should have a legend, all the *legends* being typed together on a *separate sheet*. Coloured illustrations are reproduced at the author's expense.

References. References should be numbered in the order in which they are cited in the text and listed in numerical sequence on a separate sheet at the end of the article. The numbers should appear in the text at the appropriate places using superscript numerals. In the reference list, periodicals[1], books[2], and multi-author books[3] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 R. D. Marshall and A. Neuberger, in A. Gottschalk (Editor), *Glycoproteins*, Vol. 5, Part A, Elsevier, Amsterdam, 2nd ed., 1972, Ch. 3, p. 251.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*.

Proofs. Two sets of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

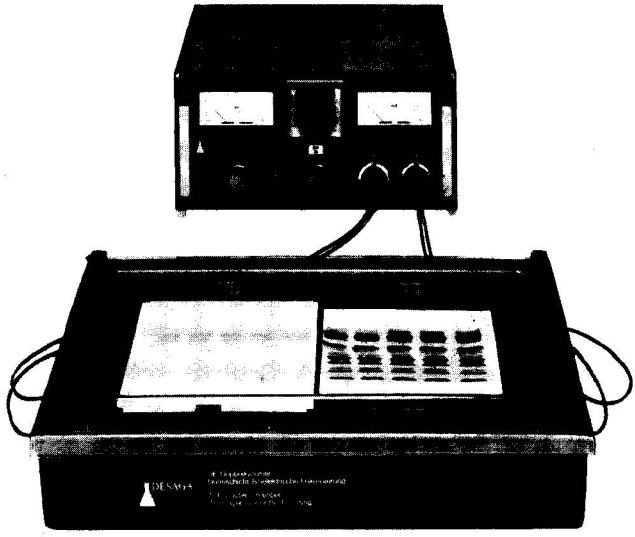
News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography, Elsevier Scientific Publishing Company, P.O. Box 330, Amsterdam, The Netherlands.

Subscription orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, Amsterdam, The Netherlands.

Publication. The *Journal of Chromatography* (including *Chromatographic Reviews*) appears fortnightly and has 13 volumes in 1974. The subscription price for 1974 [Vols. 88–100] is Dfl. 1066.00 plus Dfl. 65.00 (postage). Subscribers in the U.S.A., Canada and Japan receive their copies by air mail. Additional charges for air mail to other countries are available on request. Back volumes of the *Journal of Chromatography* (Vols. 1 through 87) are available at Dfl. 92.00 (plus postage).

Advertisements. Advertisement rates are available from the publisher on request. The Editor of the journal accepts no responsibility for the content of the advertisements.

DESAGA



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Maybe you are also interested in other separation techniques. The DESAGA TLE Double Chamber® can also be used for Thin-layer Electrophoresis, Preparative Electrophoresis and Paper Electrophoresis. You can buy this versatile apparatus at an extremely reasonable price.

Ask for product information 173.

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Founded in 1840

World Hallmark
of Thin-layer
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