Journal of Chromatography, 492 (1989) 471–538
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4783

REVIEW

PLANAR CHROMATOGRAPHIC TECHNIQUES IN BIOMEDICINE· CURRENT STATUS

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(First received February 10th, 1989, revised manuscript received April 3rd, 1989)

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0378-4347/89/$03 50

LIST OF ABBREVIATIONS

AMD	Automated multiple development
BBOT	2,5-Bis(5-*tert* -butyl-2′-benzoxazolyl)thiophene
DEAE	Diethylaminoethyl
DMA	3,4-Dihydroxymandelic acid
DRIFTS	Diffuse reflectance infrared Fourier transform spectroscopy
EDTA	Ethylenediaminetetraacetic acid
ES	Endogenous substances
ExS	Exogenous substances
FAB	Fast atom bombardment
FID	Flame ionization detection

GC	Gas chromatography
HETP	Height equivalent to a theoretical plate
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
HVA	Homovanillic acid
INT	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
IR	Infrared
LC	Liquid chromatography
L/S ratio	Lecithin/sphingomyelin concentration ratio
MS	Mass spectrometry
NAD	Nicotinamide adenosine diphosphate
NP	Normal phase
OPLC	Over-pressured layer chromatography
PC	Paper chromatography
PG	Prostaglandins
PLC	Planar chromatography
PMA	Phosphomolybdic acid
PTH	Phenylthiohydantoin
RDS	Respiratory distress syndrome
RIA	Radioimmunoassay
RP	Reversed-phase
R S D	Relative standard deviation
SIMS	Secondary ion mass spectrometry
TLC	Thin-layer chromatography
UV	Ultraviolet
VIS	Visible
VMA	Vanillyl mandelic acid

1 INTRODUCTION

The analysis of intermediates and participants in metabolic pathways should provide a clue to the site of a metabolic disturbance Endogenous substances (ES) and/or exogenous substances (ExS) must be determined. Most of the ES are present at low concentrations in the presence of large amounts of contaminants Laboratories are required to supply the clinician with rapid and valuable information from reliable laboratory tests In the study of a disease or a biochemical pathway, the assay time is not critical, but unambiguous identification and quantitation of analytes are needed. Even with the widespread use of immunoassays, chromatographic and electrophoretic techniques are involved when separation is required and/or when antibodies cannot be devel-

oped or are not specific enough It should be pointed out that chromatographic techniques approach the sensitivity of radioimmunoassay (RIA).

In this paper we shall focus on planar chromatography (PLC), which means chromatography in a thin, planar, stationary field with a length to thickness ratio often exceeding 1000 1 Initially, paper chromatography (PC) was the only planar chromatographic method, but it has been superseded by conventional thin-layer chromatography (TLC). Nowadays the number of reports on PC is sharply declining and this technique is often considered obsolete. TLC was extensively used in the period 1970–1980 As a skilful technician was needed to handle properly the large number of experimental variables, analysts looked for a fully instrumentalized technique, and high-performance liquid chromatography (HPLC) gradually replaced TLC

Excellent books appeared on column chromatography [1–3] and the current value and potential of PLC was ignored [4] However, in 1976, Zlatkis and Kaiser [5] edited a book on high-performance thin-layer chromatography (HPTLC) and soon after Guiochon and co-workers [6–9] began to publish extensive work on the performances of TLC In the early 1980s, forced-flow techniques, particularly over-pressured layer chromatography (OPLC), were pioneered by Tyihak et al. [10] and are now readily available [11,12] In recent years, major advances in TLC instrumentation have been achieved [13].

TLC evolved towards more sophisticated and more instrumentalized techniques and planar chromatography was introduced in this Journal in 1987 to take account of those significant improvements

The purpose of this review is to outline recent progress in the field, to emphasize the fact that most chromatographic techniques are complementary rather than competitive and to survey applications in biomedicine. We selected papers from a retrospective literature search through a direct link to Chemical Abstracts Service In the first part of this survey we could not quote all relevant papers which would be far beyond the scope of this review (as we discarded electrophoretic techniques), and in the Applications sections we apologize for missing references Some valuable books and reviews have appeared that are referenced in different parts of this paper.

2 EXPERIMENTAL TECHNIQUES

2 1 Stationary phases

Many types of precoated plates are commercially available. TLC plates are usually 0.25 mm thick and made with particles ca 12 μm in diameter. HPTLC plates have a smaller particle size (7 μm), a smoother surface and the layer is often thinner (0 20 mm). Advantages and drawbacks of both TLC and HPTLC plates have been thoroughly studied [14,15]. HPTLC layers necessitate appropriate dimensioning of sample volumes, high-quality application and very

precise positioning Owing to the possible lack of precise instrumentation, TLC plates are still twice as often reported in the literature as HPTLC plates

The following stationary phases are currently available: (a) polar inorganic sorbents (silica gel and, to a much lesser extent, alumina and some oxides), (b) polar organic sorbents (cellulose, polyamide), (c) bonded silicas of either polar or non-polar types (C_2, C_8, C_{18}, phenyl, cyano, amino, diol); (d) ion exchangers, and (e) complex-forming stationary phases or chiral phases for enantiomeric separations

Most of these phases are identical in their synthesis to those available in HPLC Some are specifically devoted to PLC The presence of a binder and of a fluorescent indicator may cause some slight disturbances in R_F values

2 1 1 Inorganic polar sorbents

Silica gel is the most widely used. Reports on biochemical and clinical applications with alumina are scarce and metal oxide layers are hardly ever used in this field The silica surface is capable of trapping water molecules from the laboratory atmosphere or from developing solvents, with consequential differences in the observed and published R_F values Procedures similar to current practice in HPLC (mixtures of dry and wet solvents to ensure identical surface activity) are only possible with OPLC Great care and standardization of experimental conditions are necessary and authentic samples must be co-chromatographed with the analytes to be separated in order to ensure reproducible chromatographic data

In the classical adsorption mechanism, the retention of organic functional groups is as follows hydrocarbons < aromatic hydrocarbons < halogen derivatives < ethers < esters < aldehydes < ketones < alcohols < amines < carboxylic acids In general, the adsorption technique is used for the separation of non-polar or moderately polar materials As many biologically important solutes are highly polar molecules, they are strongly retained and solvents or mixtures of solvents of high eluting strength are needed In some instances a high water content is advocated (see Section 3) This works reasonably well but a partition mechanism is involved, with consequential disturbances in the above retention order More troublesome is the substantial demixing which may occur the components of the eluent which are sorbed strongly by the sorbent sites can cause secondary fronts (F_α, F_β), and these fronts divide the sorbent layer into zones of different eluting strength The more complex the mixture of solvents, the more difficult it is to reproduce the retention and it is our opinion that a better insight into other systems, such as alkyl-bonded phases, should be investigated

In the Chromarod technique, the chromatographic support is a porous, sintered layer of finely divided glass which contains fine particles of an inorganic adsorbent within its pores The method for producing the Chromarod consists in mixing the fine particles, kneading the mixture with a small amount of slur-

rying agent, applying the slurry to the surface of a thin rod of refractory and chemically stable material and baking at 900 °C to bond the particles to each other, to the adsorbent particles and to the rod The particles form a firm, coherent layer (0 75 mm thick). Using this procedure, the observed R_F values are higher than those on conventional silica gel TLC plates

Owing to the Lewis acid properties of alumina, some interesting separations (e.g , steroids [16]) have been performed, but the use of such supports is rapidly declining. It should be pointed out that new interest in the use of alumina sorbents in HPLC is evident

2 1 2 Polar organic sorbents

Chromatography on polyamide is a process of simultaneous electron donor–electron acceptor interactions With non-polar developing solvents polyamide acts as a normal phase (NP) and with aqueous or polar eluents it behaves as a reversed phase (RP)

Cellulose for TLC is available in two forms native fibrous and microcrystalline In cellulose, most glucose units constitute crystalline areas bound into fibres by amorphous β-glucoside linkages It is assumed that the amorphous region may hold water, forming with cellulose a liquid gel that exhibits different solvation properties to those of water (similarly to liquid in micelles). A partition mechanism is responsible for the solute retention, which depends on molecular shape and size and the position of hydrophilic groups in the sample

The particle sizes of polyamide and cellulose are large, with the consequence of poor mass transfer Recently published separations on these sorbents are not very numerous

2 1 3 Alkyl-bonded silicas

These phases, which are similar to RP-HPLC packings, were introduced into HPTLC some years ago RP-2, RP-8 and RP-18 plates are readily available The extent to which these layers can be wetted with aqueous solvent mixtures is limited [17], and to overcome these restrictions RP layers which have a reproducible, lower degree of modification and hence a lower degree of hydrophobicity have been introduced These plates are called RPW (W indicates wettable by water) and permit the use of very high water contents (or even pure water) in the mobile phase RP-HPTLC plates have a considerably narrower particle size distribution and a smaller mean particle diameter than the corresponding RP-TLC plates RP-HPTLC plates have a denser and more homogeneous packing and a smoother surface structure, thus exhibiting better detection capabilities.

With the same developing solvent, R_F values on wettable RP-HPTLC plates are higher than those on RP-HPTLC or RP-TLC plates. Phenyl-bonded silicas behave very similarly to C_8- or C_{18}-bonded silicas In spite of the very wide capabilities of such alkyl-bonded packings, their use in planar chromatography

is only slightly increasing and applications in biomedicine are still not very numerous The latest review on the use of bonded phases appeared in 1986 [18]

2 1 4 Polar bonded silicas

Polar bonded phases currently available are of the amino, cyano and diol type, according to the nature of the relevant functional group

As in NH_2 packing materials for HPLC, the amino group takes the form of a short-chain alkylamino function The chromatographic properties of the amino plate are largely governed by the polar basic nature of the alkylamino group, which may enter into weak hydrophobic interactions However, the amino plate is wetted with pure water without addition of organic solvents Steroid separations can be performed with pure organic solvents [19] The lifetime of NH_2 columns in carbohydrate analysis is said to be short This problem does not occur with the plates

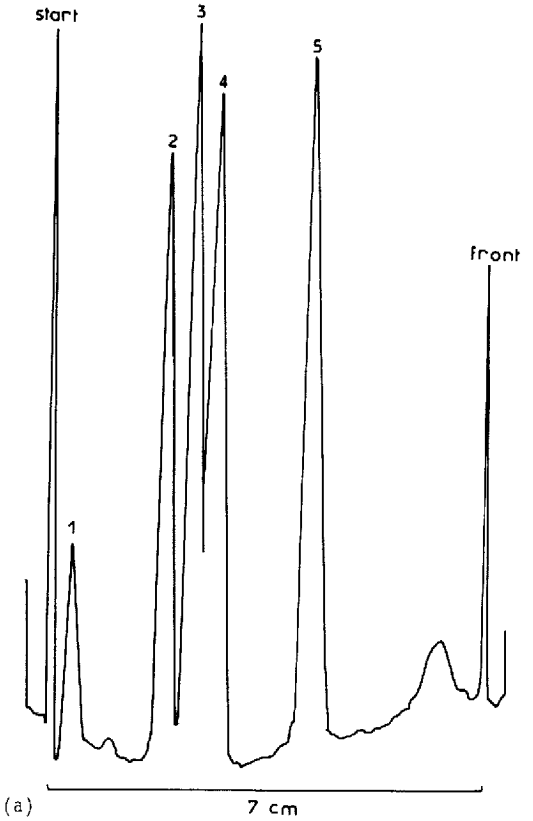
In the cyano phase, the cyano functionality is chemically bonded to the surface of the silica gel in the form of a γ-cyanopropyl group As far as polarity is concerned, the cyano phase is very close to RP-2, as was demonstrated in the separation of some corticosteroids [20] Solvent systems exhibiting very different polarities can be successfully used The cyano phase may exhibit acidic or basic properties with different eluents [21] With highly polar mobile phases, the addition of lithium chloride reduces tailing The cyano plates can be used in either the NP or RP mode, which is highly valuable in two-dimensional chromatography and overcomes the problems associated with a two-phase plate with a strip of one phase

The chromatographic behaviour of the diol sorbent is influenced by the spacer group, which is different from the propyl group utilized with cyano and amino phases $Si(CH_2)_3OCH_2CH(OH)CH_2OH$ This phase behaves in a similar way to silica gel However, the R_F values on the diol plates are higher than those observed on bare silica gel The diol surface has a lower tendency to adsorb water from the surrounding atmosphere, which leads to much better reproducibility of the retention data [22]

Examples of the potential of polar bonded phases are illustrated in Fig 1 for (a) the separation of some uridine derivatives on an NH_2 plate and (b) the two-dimensional separation of some sex hormones on a cyano plate with an NP system in the first direction and an RP system in the second

2 1 5 Ion exchangers, ion pairing

Separations through ion exchange are not widely used in PLC Polyethyleneimine and diethylaminoethyl (DEAE)-cellulose anion exchangers are the most commonly used A Dowex 50-X8 strongly acidic cation-exchange TLC plate is available in the Na^+ or Li^+ form under trade name Fixion (Chroma-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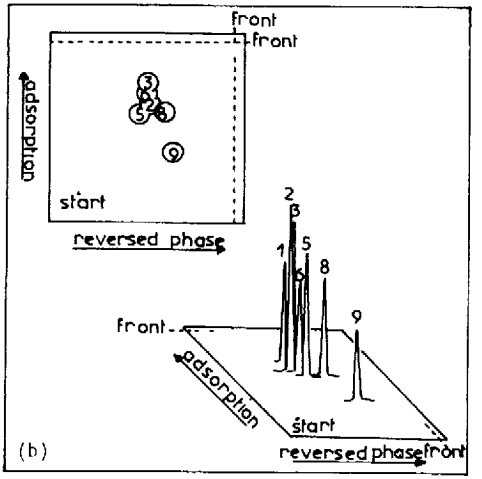
(a)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(b)

tronix, Palo Alto, CA, U S A) and has been used in amino acid analysis and separations of nucleoside derivatives [23] Unfortunately, a run requires 1–2 h in water or organic media

Owing to the basic properties of the amino group in aqueous media, the amino-bonded phase is a weakly basic anion exchanger Polyvalent anions (e g , nucleotides or sulphonic acids) are separated according to their charge, the more negative the charge, the more the solute is retained [19]

Ion-pair extraction has long been exploited for the extraction of drugs from body fluids into organic phases The ion-pairing technique is widely used in HPLC but its application in PLC is less popular The technique is easy to use when care is taken to impregnate the plate evenly by dipping or by numerous predevelopments Otherwise, the ion-pairing reagent is chromatographed and not evenly distributed in the layer Examples of applicability include the separation of urinary porphyrins [24]

2 1 6 Complex-forming stationary phases

These phases are not commercially available and must be prepared by the chromatographer They are used to carry out isomer separations It was shown long ago by Morris et al [25] that esters of *cis* and *trans* fatty acids can be separated on silica gel plates impregnated with silver nitrate Argentation chromatography has proved its usefulness in the separation of saturated from unsaturated compounds Morris [26] also demonstrated the separation of the *threo* and *erythro* forms of saturated dihydroxy esters in the form of their borate complexes on silica gel impregnated with aqueous boric acid solutions

2 1 7 Enantiomeric separations

Owing to the different biological activities of enantiomers, a direct and in expensive method for the analytical control of enantiomeric purity is important and TLC studies on the topic are now rapidly increasing

The properties of cellulose were recognized as early as 1948 when amino acids were resolved by PC [27] In 1980 Blaschke [28] reviewed chromatographic resolution on cellulose, starch and cellulose acetate Yuasa et al [29] reported the TLC separation of D- and L-tryptophan on a crystalline cellulose

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Fig 1 (a) Separation of purine derivatives Plate, HPTLC NH_2 F_{254}, solvent, ethanol-water (80 20) saturated with NaCl, migration distance, 7 cm Solutes 1 = uric acid, 2 = xanthine, 3 = hypoxanthine, 4 = guanine, 5 = adenine Detection in situ evaluation with TLC–HPTLC scanner (CAMAG), UV 254 nm Reproduced from ref 309 with permission (b) Two-dimensional separation of some sex hormones Plate, HPTLC CN F_{254}, solvent 1 (adsorption), light petroleum (b p 40–60 °C)–acetone (65 35), solvent 2 (reversed-phase system) in the orthogonal direction, acetone–water (40 60), development length, 8 cm in both directions Solutes 1 = progesterone, 2 = 5-dehydroandrosterone, 3 = pregnenolone, 4 = 4-androsten-3,17-dione, 5 = oestrone, 6 = androsterone, 7 = testosterone, 8 = oestradiol, 9 = oestriol Detection solutes 1, 4 and 7, UV 254 nm, solutes 2, 3, 5, 6, 8 and 9, spraying with $MnCl_2$–H_2SO_4, heating for 5 min at 100 °C, UV 366 nm Reproduced from ref 20 with permission

plate It seems that cellulose derivatives have a greater resolving capability The morphology of the cellulose triacetate particles changes drastically during prolonged acetylation Monocrystalline triacetylcellulose plates are stable in aqueous solvents and are resistant to dilute acids and alkalis. Excellent separations on this support have been reported [30] Tricinnamate, trisphenyl carbamate and tribenzyl ether derivatives have been developed by Ichida et al [31] and exhibit high enantioselectivity in HPLC but apparently have not yet been applied in TLC.

Cyclodextrins are cyclic oligoglucose molecules that resemble a truncated cone with both ends open. The hydrophobic cylindrical cavity can include an organic host molecule and the glucose units give a chiral environment. Bonding of cyclodextrins to silica was studied by Alak and Armstrong [32], who separated dansyl derivatives of amino acids on a 20 cm × 5 cm β-cyclodextrin-bonded TLC plate Several geometric and structural isomers have also been separated with these plates A discussion of the mechanism of separation, structural parameters, solvent effects and binder effects was presented by Ward and Armstrong [33]

Ligand exchange was introduced into LC by Davankov et al. [34] and extended by Gubitz et al [35] to silica-based stationary phases. TLC application of this concept was developed by Gunther et al [36] and Gunther [37]: an RP plate is dipped into a 0 25% copper(II) acetate solution and dried, then im-

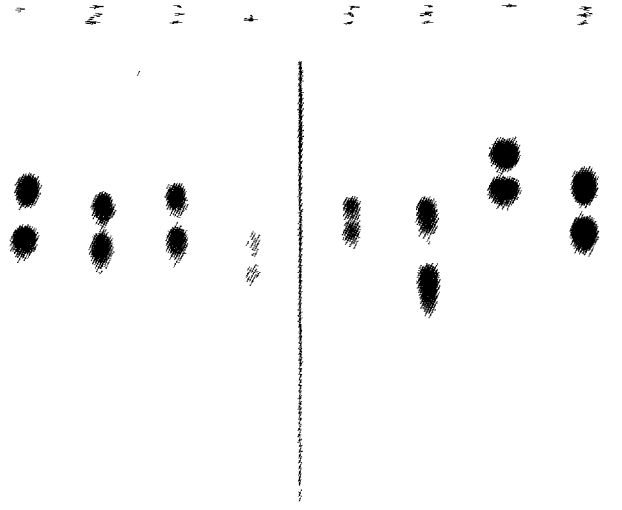


Fig 2 Photograph of a thin-layer chromatogram of proteinogenic amino acids on Chiralplate Spots 1 = phenylalanine, 2 = valine, 3 = isoleucine, 4 = proline, 5 = methionine, 6 = glutamine, 7 = tyrosine, 8 = tryptophan Eluent methanol-water-acetonitrile (50 50 30) Migration distance, 10 cm Reproduced from ref 37 with permission

mersed in a 0 8% methanolic solution of (2*S*,4*R*,2′*RS*)-N-(2′-hydroxydodecyl)-6-hydroxyproline, which is the chiral selector

Differences in the retention of two optical antipodes are caused by dissimilar stabilities of the diastereoisomeric copper complexes built up from the selector and the enantiomers to be separated [38,39] These plates are available as Chiral Plates (Macherey & Nagel, Duren, F R G) and CHIR (Merck, Darmstadt, F.R.G.), and dipping is no longer required In this mode, underivatized amino acids are resolved (Fig 2), the L-enantiomer exhibiting a higher R_F value than the D-enantiomer With dipeptides the enantiomer with the C-terminal L-configuration always has a lower R_F value than that with the C-terminal D-configuration

Chiral plates can be used with forced-flow planar techniques with some care such as a prerun to achieve better wettability of the plate with the mobile phase [40] The solvent used for the prerun has to be selected so that the compounds to be separated do not migrate and it should be a component of the mobile phase As new phases are appearing in HPLC it may be expected that new types of chiral layers will also appear in TLC, such as chiral crown ether-bonded packing or metal-modified cyclodextrins Chiral separations of acids and amines using ion-pair chromatography in an organic phase of low polarity have been successfully utilized in HPLC and may provide high stereoselectivity [41]

2 2 *Sample preparation*

Interfering substances can affect the reliability and sensitivity of a PLC system Solid-phase extraction with selective elution has reduced this problem and much time is thus saved Sample volumes from 10 to 500 μl can be treated on conventional organo-bonded silica packed in a small cartridge (Sep-Pak type) Liquid–liquid extraction on high-surface-area porous diatoms is also performed In some instances repeated solvent extractions or even more complex procedures are required More information is given in the Applications sections (e.g , phospholipids, Section 3 2) Use of preconcentration zone plates greatly facilitates the sample clean-up and pretreatment can be carried out in the preadsorbent zone In the same way prechromatographic derivatization can be carried out in situ, thus allowing a better separation of the solutes of interest with the consequence of retaining the interfering solutes on the preadsorbent zone or in the lower part of the plate A review on types of in situ prechromatographic derivatization reactions appeared recently [42]

2 3 *Sample application*

A wide range of autospotters capable of applying solutes as spots or streaks are now readily available These devices are fully microprocessor-controlled

Handling of sample working volumes have been re-examined recently by Kaiser [43]

2 4 *Development*

Development chambers have received much attention from researchers but very little from users Three geometric arrangements are possible in conventional TLC or HPTLC circular, anticircular and linear There is no definite advantage of circular or anticircular modes PLC has a unique capability, viz , two-dimensional development in orthogonal directions with a combination of two different phase systems This mode offers a surprisingly large spot capacity The latest review on applications of two-dimensional TLC appeared in 1983 [44]

In a classical TLC run, the solvent ascends the layer by means of capillary forces and the distance travelled by the solvent front (Z) and the time (t) are related by the relationship $Z^2 = kt$ The presence of a gas phase dramatically affects the solvent migration [45] through adsorption or evaporation, and Guiochon and Siouffi [46] derived a complete set of equations for every situation They proposed to plot Z^2/t versus t to check the extent of deviations from a quadratic law The main drawback of the classical development mode is that the optimum velocity of the solvent is reached within a very short period of time

Forced-flow systems appeared in which the gas phase is eliminated and proportionality of Z versus t is obtained In rotation planar chromatography the mobile phase migrates with the aid of centrifugal force A commercial instrument is available (Rotachrom, Zurich, Switzerland) for preparative and analytical purposes [47] Major advances in the field came from the OPLC technique, which has been improved year by year and can be used with plates or flat beds of any size The layer is covered with an elastic membrane under a low or high external pressure of water An additional micropump delivers the solvent to the chromatographic bed, which ensures a constant flow-rate [10] Injection of sample solutes can be performed prior to development, as in conventional TLC, or when the flat column is equilibrated All development procedures (circular, anticircular, linear, multilayer) are possible In the linear mode the migration of eluent is achieved by placing a narrow plastic sheet on the layer or making a narrow channel in the layer beyond the position of the eluent inlet The function of the eluent trough is to direct the eluent and to form a linear eluent front

The efficiencies are comparable to those obtained in HPLC [12] and it has been demonstrated that the mean HETP (height equivalent to a theoretical plate) values are almost independent of the retention characteristics of the solutes [48] However, in forced-flow systems the variance of the injection

device is critical [49] OPLC is used as a model for HPLC separations especially with ion-pair systems [50] and gradient elutions are possible

One of the main advantages of PLC is the capability of checking the separation and quickly changing solvents In sequence TLC (e g , Mobil R_F chamber from Scilab, Therwil, Switzerland), the supply of solvent to the plate layer is fully variable in space and time and two-dimensional chromatography in opposite directions can be carried out [51] Soczewinski [52] developed an equilibrium sandwich TLC chamber for continuous development and gradient elution A new concept is the automated multiple development (AMD) technique [53], which is a fully automated instrument and represents a very improved version of programmed multiple development [54] Unlike HPLC, the gradient starts with the more polar solvent and the polarity is then decreased The shortest developing distance is employed with the most polar eluting solvent and the longest distance with the most non-polar solvent Evaporation of the solvent from the layer is carried out by vacuum, no heat is applied which could provoke the formation of artifacts Apparently no report on the use of AMD for the class fractionation of complex substances, as found in biomedical analysis, has been published

2 5 Detection and identification of separated substances

In classical TLC, detection is a static off-line procedure Coloured or fluorescent spots are easily detected visually under white or UV light Some solutes exhibit native fluorescence characteristics on UV irradiation (e g , porphyrins) and are detected at very low levels In most instances microreactions are used to enhance the detection by transformation of the functional groups Reactions are carried out by either spraying the reagent onto the plate or dipping the layer in the reagent solution In both instances subsequent heating is necessary The non-uniformity of the spray may dramatically affect the linearity of the response On the other hand, dipping can damage the layer or some amount of an analyte may be dissolved in the solution Post-chromatographic derivatizations for TLC have been extensively reviewed [55,56] Measurement of the in situ fluorescence of separated spots has many advantages over in situ UV absorbance measurement With the careful selection of excitation and emission wavelengths, the sensitivity is often increased by a factor of 100–1000 Fluorescence signal enhancement of 10–200 fold was observed when the plate was dipped in a solution of a viscous liquid such as paraffin [57] Baeyens and Lin Ling [58] recently produced a useful table of derivatizing reagents for the fluorescence labelling of functional groups Very promising is fluorescence line narrowing spectrometry, which requires cooling of the TLC plate to 10–50 K prior to analysis [59] A very impressive detection of polycyclic aromatic hydrocarbon–nucleoside adducts was recently reported [60]

Combinations of chromatographic techniques and enzyme reactions give

more specific analyses of the compounds in a body fluid For example, steroids in urine can be detected using 3β-hydroxysteroid oxidase [61] (see also Sections 3 2 and 3 3)

Detection of compounds eluted on a Chromarod can be performed either by flame ionization detection (FID) [62] or by direct insertion into a thermoionic detector [63] These detection techniques are mainly used with lipids [64]

In recent years, significant advances have been made in the coupling of PLC with mass spectrometry (MS) and infrared (IR) spectrometry

Transfer of the solute from a plate to a mass spectrometer is a time-consuming procedure Some devices, such as the Eluchrom (Camag, Muttenz, Switzerland), permit the recovery of the separated samples Glycosphingolipids are extracted from the layer with methanol, permethylated, purified on a Sep-Pak cartridge, dissolved in 3-mercapto-1,2-propanediol and analysed by fast atom bombardment (FAB) MS [65] Ramaley et al [66] reported a method for obtaining a chromatogram and mass spectra simultaneously The method involves heating part of the TLC plate with an incandescent filament or pulse laser while moving the TLC plate in order to desorb sequentially the separated components, which are introduced into a mass spectrometer together with the reagent gas for chemical ionization The method has been used with lipids The current trend is towards FAB–TLC without an extraction procedure a TLC plate can be placed into the cavity of a helium–neon laser which serves as a probe An argon pump laser overlaps with the probe beam at the chromatogram spot [67] Another soft ionization technique is secondary ion mass spectrometry (SIMS), which can yield a strong signal with peptide molecules, developed by Busch [68] A modular instrument for direct TLC–SIMS is now on the market

IR spectroscopy involves the transfer of the TLC eluate to an IR-transparent substrate [69] prior to measurement of its Fourier transform diffuse reflectance IR spectrum (Analect, Irvine, CA, U S A) Diffuse reflectance Fourier transform spectroscopy (DRIFTS) permits both quantitative and qualitative characterization of a TLC spot with one measurement A general paper on TLC–DRIFTS appeared in 1988 [70]

2 6 Quantitation

Scanning densitometry is still the most efficient procedure for obtaining reliable quantitative data Some difficulties arise, as the densitometric quantitation of spots depends on the mass of material in the spot but also on the area which the spot occupies Modern densitometric scanners are linked to personal computers and are equipped for automated peak location, multiple-wavelength scanning and spectral comparison of fractions and are capable of measurement in any mode (reflectance, absorption, transmission, fluorescence) A laser has a higher spectral intensity than a lamp source and can be confined to zones of 5–10 μm The sensitivity is dependent on the nature of

the layer (polyamide or silica) and picomoles of solutes are detected [71] However, a soft laser is required in order to avoid laser-induced chemical reactions With ultraviolet–visible (UV–VIS) or fluorescence analysis the detection limits are 0 1–1 ng, which compare well with current practice in HPLC For radiolabelled compounds, new computer-assisted systems based on radiation imagery, linear analysers and the beta camera offer better resolution and more accurate quantitative data

Videodensitometry is now emerging The video-based image analyser utilizes a TV camera coupled to a suitable imaging device to produce an electronic video image, which is then suitably processed Camera tubes are also called vacuum image sensors and were introduced by Gianelli et al [72], Pongor [73], Prosek et al [74], Gonnord [75] and Belchamber et al [76] Solid-state sensors are usually photodiodes and exist in linear form or as matrix devices A matrix sensor with 2048×2048 diodes has recently been introduced and solid-state sensors are considered to be the future [77] From a comparison between video and classical densitometry, the advantage is still in favour of the latter mode [78] OPLC is suitable for both on-line and off-line detection In the off-line mode, classical TLC scanning densitometry can be utilized In the on-line mode, the eluites are measured in the drained eluent by connecting a flow cell detector to the eluent outlet [12] in a similar manner to that in HPLC procedures

2 7 Trends

Selection of the 'best' solvent composition for the mobile phase is a major task Optimization procedures which are now popular in HPLC are entering the domain of TLC

The Prisma model of Nyiredy et al [79] is derived from statistical mixture design and consists of an unlimited number of triangular solvent diagrams stacked to form a prism Inside the triangle it is possible to define several different combinations of solvents and the vertical axis corresponds to increasing solvent strength The three best solvents resulting from preliminary experiments are chosen to determine the corners of the prism The solvent strength of the mixture is then adjusted by dilution with *n*-hexane In the next step at optimum solvent strength the selectivity is optimized by choosing three points near the corners of the triangle This model has received much attention in both TLC and OPLC [80] In PLC the more readily accessible measure of the quality of a separation is the distance between the different spots De Spiegeleer et al [81] pointed out that a resolution-based criterion would be impossible to handle owing to the lack of information on spot widths The ideal separation would have q components equally spaced The ideal value for the jth ordered R_F value would be $(j-1)/(q-1)$ If the R_F values are ordered in ascending order, Bayne and Ma [82] defined the response function as

$$D=\left\{\sum_{j=1}^{q}[(R_f)_j-(j-1)/(q-1)]^2\right\}^{1/2}$$

which must be minimized A further refinement is to add a term that measures spread represented by the standardized fourth central moment of the R_F values In the proposed procedure, the optimization of the separation response was based on a Scheffe polynomial estimated from 25 experimental runs, which is far too many

In two-dimensional PLC, Gonnord et al [83] have defined two functions, the aim being to maximize the sum of the squares of all the possible distances between any pair of spots (the D_A function) and to minimize the sum of the inverse of the squares of the distances between pairs of spots after elimination of the unresolved pairs (the D_B function)

$$D_A=\sum_{i=1}^{k}\sum_{j=i+1}^{k}[(x_i-x_j)^2+(y_i-y_j)^2]$$

$$D_B=\sum_{i=1}^{k}\sum_{j=i=1}^{k}\frac{1}{(x_i-x_j)^2+(y_i-y_j)^2}$$

where x and y are the distances travelled by solutes i and j in one development (x) and orthogonal development (y)

Nurok and co-workers [84,85] proposed a planar response function which is derived from the above D_A and D_B functions and developed an optimization scheme for the separation of standard steroids with either continuous or two-dimensional development

In spite of the capacities of two-dimensional PLC, the coupling of different chromatographic techniques may solve some very difficult separations Combination of HPLC and TLC gives access to those features that are unique to each technique Brinkman [86] has pointed out that a thin layer can be used as a storage plate to apply detection principles that are not readily suitable in HPLC (see fluorescence line narrowing) French and Shelly [87] described a nebulizer interface for combining micro-HPLC with HPTLC and Hofstraat et al [88] recently published some quantitative determinations with a coupled HPLC–TLC system

2 8 Planar or column chromatography

PLC is often critically compared with gas chromatography (GC) and HPLC This is a matter of endless dispute, but some facts are obvious

With the exception of capillary zone electrophoresis, no separation technique can compete with capillary GC in terms of the number of theoretical plates, N, as 100 000 plates are routine laboratory practice Most available

HPLC columns can provide 1000 plates/cm (15 000 plates with a 15-cm column) and only 3000–4000 are attainable on the whole plate under the optimum TLC conditions [7] OPLC fills the gap but is still less efficient than HPLC It should be pointed out that fully off-line OPLC exhibits the lowest HETP values and fully on-line OPLC the highest values

A peak capacity of 100 requires an HPLC column of 40 000 theoretical plates, which is more than classical columns can produce but is very easily achieved in two-dimensional PLC With respect to biomedical analysis, a two-dimensional PLC development is easier to handle than a column-switching technique

In terms of speed of analysis, a single 10×10 cm TLC plate permits the simultaneous analysis of fifteen samples, whereas runs have to be performed sequentially in GC or HPLC PLC is only very cheap when qualitative results are needed When reliable quantitative data are required, PLC is as expensive as column chromatography GC and HPLC are fully automated, whereas PLC still requires manual transfer of the plates In OPLC the edges of the layer must be pretreated Since in conventional TLC the planar chromatographic bed is used only once, there is no need for any precautions and solvents containing a strong base (e g , ammonia) can readily be used, whereas they would quickly destroy an HPLC column

Comparison of minimum detectable concentrations is difficult According to Brinkman [86], the ppb level is attainable with GC or HPLC whereas the ppm level is standard in PLC (with some remarkable exceptions) The advantage will be on the side of HPLC as it is easier to detect solutes in transparent cells than on non-transparent layers Table 1 lists some of the main features of HPLC and PLC and is far from exhaustive In the authors' opinion, complementary rather than competitive chromatography provides the best answer to analytical requirements

3 APPLICATIONS TO ENDOGENOUS SUBSTANCES

3 1 Lipids

TLC is eminently suitable for lipid quantitative analysis, the most general technique for obtaining a qualitative profile of a total lipid extract is TLC Lipid measurement is of the greatest interest in blood serum, faeces, tissues and amniotic fluid The investigation of serum lipids, mainly cholesterol ester subfractions, has clinical importance in the diagnosis of hepatic diseases and lipid metabolism disorders Hyperlipaemia has an outstanding place among the risk factors of cardiovascular diseases Dermatologists are interested in sebum analysis as some unique features have been evidenced (waxes, squalene, long-chain alcohols) The Vernix caseosa of the human newborn contain diol lipids which are not found in the adult The amounts of extractable lipids may be abundant or very small In studies of cells in culture, the concentration of

TABLE 1

SOME FEATURES OF PLC AND HPLC

PLC	HPLC
Advantages	
Large number of samples analysed simultaneously	Very high efficiencies
Two-dimensional capacity	On-line data acquired in a few seconds
Preservation of the total chromatogram	Full automation
Short time of analysis	Easy gradient elution
Multiple detection capabilities	High-technology LC–MS currently available
No solvent requirement for detectability of solutes	Sophisticated data acquisition and handling
Sample pretreatment on the plate	
Drawbacks	
Presence of a gas phase (except in OPLC)	One sample analysed per run
Off-line detection (except in OPLC)	Column lifetime
Limited column length	Last eluting peaks difficult to check
Limited injection volume	Solvents compatible with detectors
Combination with spectrometry more difficult than with HPLC	

most lipids is of the order of femtomoles (10^{-15} mol) per cell The chromatography of lipids and particularly the planar chromatography of lipids is very well documented, as many efforts have been made and successful achievements have been obtained in the field of edible oils and fats A general review on lipid analysis appeared in this Journal [89], and a book on the chromatography of lipids in biomedical research appeared in 1987 [90]

Polar lipids (phospholipids and sphingolipids) and non-polar or slightly polar lipids (glycerides and sterols esters) are the two main classes, but many subdivisions exist and class fractionation is one of the major tasks of the analyst

3 1 1 Class fractionation

The extraction procedure of Folch et al [91] with chloroform–methanol is generally advocated, with some slight modifications Extraction of lipids from total plasma or cerebrospinal fluid is performed according to the procedure of Nelson [92] or Mitchell et al [93] In biomedicine, one class of compounds generally requires attention (e g , cholesterol and cholesterol derivatives in blood serum lipids, phospholipids in amniotic fluids) For this purpose, preparative class fractionation is often carried out by PLC prior to GC or HPLC analysis of individual classes

PLC is unique as successive developments can be performed with different solvents to enhance the resolution between the different classes A single run

may be sufficient, but a single solvent does not generally resolve lipid classes Triglycerides can be separated with benzene or chloroform, but different classes of glycerides are only separated with binary or ternary mixtures (Table 2) Chromatography of faecal lipids on Chromarods is carried out with benzene–chloroform–formic acid (35 15 1) and gives separation in the order cholesteryl esters, triglycerides, free fatty acids, cholesterol, diglycerides, monoglycerides, phospholipids [94]

Two types of procedures are recommended in the literature: starting with a strong eluting solvent with the use of a less polar solvent in a second or a third run, or the reverse

In the first mode, the procedure of Murawski et al [95], checked in a recent book [96], consists of a 1-cm run with chloroform–methanol–water (75 25 4) followed, after drying, by migration with chloroform to the middle of the plate and then hexane–chloroform (30 10) to the upper edge of the plate Slight modifications have been proposed, such as changes in solvent proportions (65 25 4) [97] or the addition of acetic acid [98], acetic acid–formic acid [99] or ethyl acetate–acetic acid [100] Different development distances (e g , 4 5 cm) can also be applied

For the second development, hexane–acetone (100 1) [95], hexane–diethyl ether–acetic acid (35 15 1) [98] or hexane–diisopropyl ether–acetic acid (65 35 2) [100] have also been advocated With the last system, separation of lipids into fourteen fractions (five phospholipid fractions and nine neutral lipid fractions) is achieved without the need for a third development In a rapid analysis of cellular lipids a suspension of cells grown in sodium [^{14}C]acetate solution is applied directly to silica gel plates and development is performed first with chloroform–methanol–water–acetic acid (25 15 2 4) and then with

TABLE 2

R_F VALUES OF MAIN CLASSES OF LIPIDS ON SILICA GEL

Mobile phases I = benzene, II = chloroform, III = trichloroethylene–acetonitrile (85 15, v/v), IV = heptane–diethyl ether–acetic acid (70 27 10, v/v/v)

Solute	R_F			
	I	II	III	IV
Fatty acids	0 00–0 04	0 05–0 07	0 25–0 29	0 32–0 35
Fatty acid esters	0 34–0 36	0 60–0 62	0 70–0 71	0 50–0 54
Monoglycerides	0 01–0 03	0 02–0 06	0 14–0 16	0 09–0 10
Diglycerides	0 02	0 07–0 015	0 52–0 61	0 22–0 25
Triglycerides	0 18–0 22	0 57–0 60	0 77–0 80	0 40–0 50
Sterol esters	0 60–0 64	0 70–0 75	0 80–0 83	0 72
Phospholipids	0 00	0 00	0 00	0 00

hexane–diethyl ether–acetic acid (35 15 1) Subsequent autoradiography quickly identifies the separated fractions [98]

The first development can be carried out with methanol to 3 cm from the origin After drying, a second development is carried out with trimethylpentane–diethyl ether–acetic acid (75 25 2) In the first solvent, phospholipids move slightly above the origin whereas other lipids move to the second front [101,102] The second solvent resolves the mixture into its major lipid classes In the microanalysis advocated by Yao and Rastetter [103], non-polar lipids are separated with two solvent systems (a) benzene–diethyl ether–ethanol–acetic acid (60 40 1 0 05) followed, after drying, with (b) hexane–diethyl ether (47 3) Sphingo- and phospholipids are separated with (c) methyl acetate–propanol–chloroform–water–0 25% potassium chloride (25 25 25 10 9), then with solvent a Total lipids could be separated with solvent c followed by hexane–diethyl ether–acetic acid (60 40 0 23) and finally with hexane

Other solvent systems of the same type with mixtures of propanol, methanol, chloroform, water and acetic acid can be found in an extensive literature

Conversely, a sequence of solvents of increasing eluting strength may be used Major lipids of tissues and lipoproteins are separated by automated HPTLC on silica gel with three solvent systems (a) hexane–heptane–diethyl ether–acetic acid (126 37 37 2) for neutral lipids, (b) chloroform–heptane (20 30) for cholesteryl esters and (c) methyl acetate–propanol–chloroform–methanol–43 m*M* potassium chloride (25 25 25 10 9) [104]

On developing the plate first with light petroleum–diethyl ether (90 10) and then with light petroleum–diethyl ether–acetic acid (400 100 1) but to a second front 5 cm below the first front, the polar lipids remaining at the origin consist almost entirely of phospholipids [105]

Excellent separation of lipid classes was achieved by Bounias [106], who carried out the first migration with benzene to 12 cm, followed by a 7-cm migration with trichloroethylene–acetonitrile (Fig 3) The advantage is the use of simple solvents without the need for acetic acid, which in many instances is highly demixing with the consequence of a 'β front' on the plate

In a sample of human gall-bladder bile, separation of bilirubin, cholesterol, bile salts and phospholipids was achieved with chloroform–light petroleum–methanol–acetone (60 20 10 10) developed to 10 cm and acetone–water (50 50) developed to 5 cm The separation was then applied to Chromarods for quantitative purposes (cholesterol, total bile salts, phospholipids) [107]

Dual-phase plates have not received much attention, which is surprising Jee and Ritchie [108] separated major triglycerides from fatty acids, diglycerides and phospholipids with dual-phase Whatman (Clifton, NJ, U S A) KCS5 plates and three developments with acetone–acetonitrile (40 1) on the reversed-phase lane Saturated and unsaturated triglycerides were then further separated on the silica part of the plate impregnated with silver nitrate by dipping

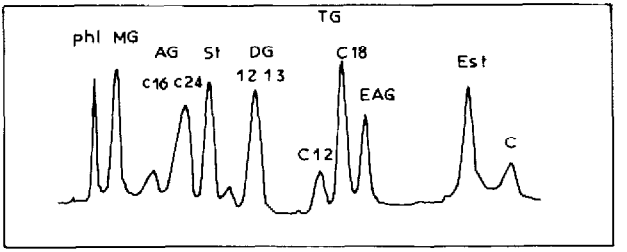


Fig 3 Lipid class separation on a silica gel plate with two sequential migrations (a) migration with benzene for 12 cm, (b) migration with trichloroethylene-acetonitrile (80 20) Solutes (in increasing order of retention) C = cholesterol, Est = cholesteryl esters, EAG = fatty acid esters, TG = triglycerides, C18 = tristearin, C12 = trilaurin, DG = diglycerides, 13 = 1,3-diglycerides, 12 = 1,2-diglycerides, St = stearic acid, C24 = arachidonic acid, C16 = palmitic acid, MG = monoglycerides, phl = phospholipids Detection charring with H_2SO_4

In three-way TLC [109], precoated silica gel plastic plates are cut to give a square with a strip attached at the top right-hand corner In the first run a lipid extract is applied to the square portion just below the strip and chromatographed with diisopropyl ether–methanol–acetic acid (50 0 6 0 8), which moves non-polar lipids on to the strip, leaving polar lipids at the origin. The strip is then cut off and separation of the lipids on the strip and the remaining square portion of the plate is completed by conventional one-dimensional development with chloroform–methanol–acetic acid–water (25 8 8 1) or, better, by two-dimensional development [benzene–light petroleum (40 60)] The method has been applied to the analysis of lipids in human bronchoalveolar lavage fluid but quantitation has not been performed

Lipid classes of blood serum from liver disease patients were separated by OPLC on silica gel with toluene–carbon tetrachloride (1 3) as the mobile phase followed by toluene alone, which prevents the use of acetic acid Pick et al. [110] applied a combination of HPLC and OPLC, HPLC was used for the semi-preparative class fractionation of underivatized lipids and OPLC was then utilized for the analytical separation of the individual glycolipids

3 1 2 Analysis of non-polar or slightly polar lipids

Quantification of the individual components of this lipid class is of the greatest interest for the assessment of assimilation diseases Cholesteryl esters, triglycerides and free cholesterol are well separated on silica gel HPTLC plates with hexane–heptane–diethyl ether–acetic acid (63 18.5 18 5.1) (Fig 4a). Further development with heptane–chloroform (60 40) permits the separation of cholesteryl esters according to the number of double bonds (Fig 4b) [104] Separation of triacylglycerol into subclasses containing straight- and branched-chain fatty acyl moieties is achieved by HPTLC on silica gel plates using hexane–diethyl ether–acetic acid (90 12 1 5) as the developing solvent [111]. Free cholesterol is always well separated from the other lipids and can

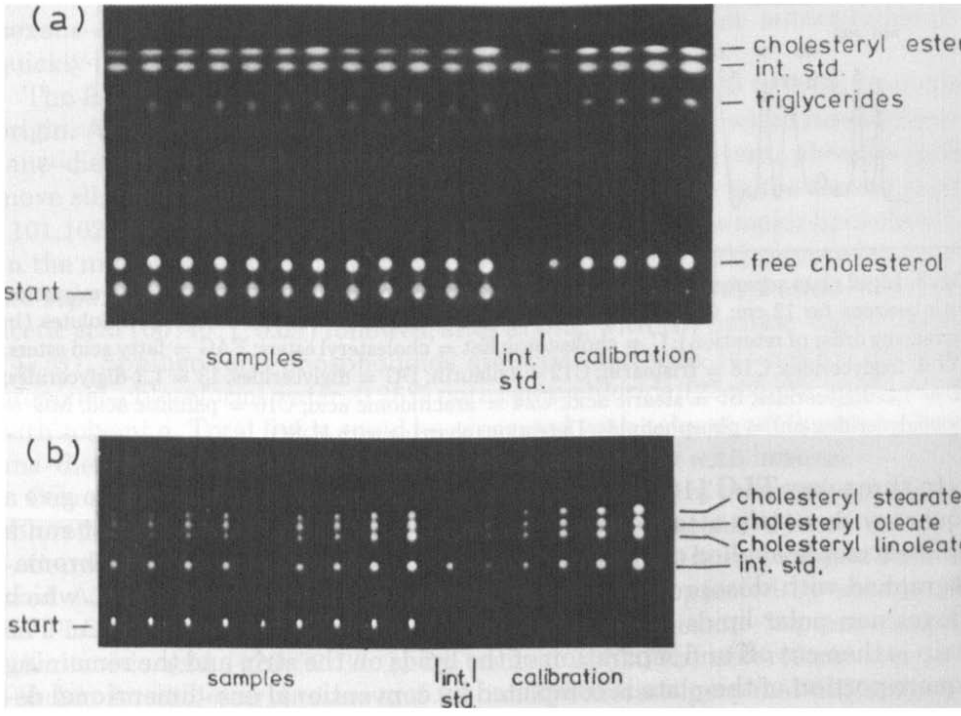


Fig 4 (a) Separation of the neutral lipids on HPTLC plates in *n*-hexane–*n*-heptane–diethyl ether–acetic acid (63 18 5 18 5 1, v/v) The external standards, consisting of cholesterol, triolein, cholesteryl formate (internal standard) and cholesteryl linoleate for calculating the calibration graphs are applied on the first five tracks (cholesterol 0 008–0 155 nmol, triolein 0 018–0 36 nmol, cholesteryl formate and cholesteryl linoleate 0 013–0 26 nmol) Track 6 contains the internal standard in the concentration as added to the samples (0 014 nmol per spot) The samples are applied on the remaining tracks For documentation purposes, the HPTLC plates have been overloaded with the samples (b) Separation of the cholesteryl esters (cholesteryl stearate, cholesteryl oleate, cholesteryl linoleate and the internal standard cholesteryl formate) on HPTLC plates in *n*-heptane–chloroform (60 40, v/v) The external standards are applied in masses between 0 039 and 0 26 nmol The concentration of the internal standard applied on track 5 is 0 104 nmol per spot For documentation purposes, the HPTLC plates have been overloaded Reproduced from ref 104 with permission

be easily quantitated Simple diagnosis of acid lipase deficiency (Wolman's disease) by detection of cholesteryl ester accumulation in the fibroblasts has been proposed, using TLC separation and Nile Red staining [112]

In the diagnosis of cerebrotendinous xanthomatosis [113], only 100 μl of serum are needed. Cholesterol was converted into α- and β-epoxides with *m*-chloroperbenzoic acid and separation from cholestanol was effected by RP-TLC Quantitation of cholestanol was carried out by spraying with phosphomolybdic acid (PMA) and subsequent densitometry The method correlates well with GC measurements and is useful for primary diagnosis [114]

Lipids from patients with alcoholic liver disease were analysed by TLC with specific assays for the major lipid classes, and it was demonstrated that alcoholic fatty liver is accompanied by a major increase (up to 50-fold) in triglycerides and a smaller (2–3 fold) increase in cholesteryl esters [115] As was emphasized in a review of lipid analysis by Kuksis [90], preparative TLC with subsequent GC analysis is the method of choice

In the quantitative determination of free cholesterol and cholesteryl esters in skin biopsies [116], the two classes are separated by TLC, then a silylation or transesterification with sodium methoxide reagent is performed with subsequent analysis by capillary GC As was expected, patients with atherosclerosis show higher values of cholesterol than normal In the investigation on the sterol pathway, Pill et al [117] used silver nitrate-impregnated plates to isolate radioactively labelled cholesterol prior to GC–MS However, it has been claimed that the separation and quantitation of fatty acids, sterols and bile acids in faeces can be carried out by GC as butyl acetate derivatives with no overlapping, obviating the need for prior separation of each class by TLC or column chromatography [118] Scraping the plates is tedious and the direct analysis of lipids on TLC plates by matrix-assisted SIMS does not require scraping or elution [119] Only 1 μg of lipids is necessary and no interference from the adsorbent occurs Isolation of cholesteryl esters by preparative TLC followed by alkaline hydrolysis and enzyme detection with cholesterol esterase (Boehringer, Mannheim, F R G) was carried out by Wolff [120], who claimed that contrary to other reports in the literature, cholesterol esterase from Boehringer has no specificity for the length of fatty acid chains or the degree of unsaturation The kit can therefore be used for the detection of cholesterol esters from sources other than human serum

Triglycerides constitute a sizeable percentage of sebum, the role of which is well established in the aetiology of many skin disorders, principally acne vulgaris In the analysis of sebaceous wax ester secretion rates in men and women aged from 15 to 97, overnight absorption of lipid into a layer of bentonite clay was carried out and subsequent TLC performed [121] The results suggest that sebum secretion declines by 20–30% per decade with a higher decrease for women A sebum-absorbent tape (Sebu Test Strip) was compared with the hexane extraction technique and free fatty acids, cholesterol, triglycerides, waxes and cholesterol esters were separated and determined by TLC [122] Collected human skin surface lipids were subjected to preparative TLC using silica gel H and successive development with hexane–benzene (50 50) and hexane–diethyl ether–acetic acid (70 30 1) [123] A mixture of trichloroethylene and acetonitrile (90 10) is claimed to be able to separate triglycerides according to the degree of unsaturation [124], but the resolution between triolein and tristearin is poor Argentation chromatography is more suitable [125]

Owing to the limited efficiency of TLC, the separation of fatty acids or fatty acid esters according to the chain length is tedious Derivatization into *p*-

bromophenacyl esters or the formation of isatin derivatives [126] followed by RP-TLC on C_{18} plates with methanol–water (88 12) yields a poor separation in comparison with GC profiles

Quantitative lipid determination by TLC is still a matter of dispute Glycerides are measured in hospital laboratories by spectrophotometric or enzymatic procedures and TLC may be superior in performing a class separation into mono-, di- and triglycerides and subsequent quantitation. The lipids are usually rendered visible and subsequently quantitated by either charring or staining Both methods have drawbacks loss of carbon by conversion to carbon dioxide (charring) or fading of the colours of spots (staining) Exposure of the plate to iodine vapour in a sealed tank was considered as a qualitative method It was converted into a quantitative technique by measuring the rate of decolorization of a solution of cerium(IV) by arsenic(III) [127] However, the method requires scraping of the plate and is time-consuming

An inter-laboratory trial on the quantitation of cholesterol by TLC and HPTLC was initiated by Dammann et al [128] The conclusions were that very precise and accurate analytical results are obtained even allowing a free choice of analytical conditions The mean values found was ca 10 mg/l with an excellent standard deviation of reproducibility (0 76–1 23 mg/l) Fluorescence measurements after derivatization with manganese(II) chloride is by far superior to the same derivatization followed by reflectance mode scanning at 546 nm FID, derivatization with anisaldehyde or sulphuric acid and fluorescence measurement are comparable and yield the same results A limit of detection of 0 02 μg for sterol and sterol esters was claimed [129] by dipping in copper(II) sulphate and densitometry FID with an Iatroscan TH10 (Iatron Laboratories, Tokyo, Japan) has been considered unreliable [130], but is very precise when care is taken to ensure maximum reproducibility [131,132] Quadratic regression lines are obtained by plotting concentrations versus peak areas [94] However, linear regression analysis produced correlation coefficients of 0 998 for cholesterol over the concentration range 0 25–8 0 μg/ml The method is considered to be so precise that it could be used to calibrate instruments such as the Infraanalyser (Technicon, Tarrytown, NY, U S A), which performs the quantitation of serum lipids by near-infrared reflectance spectroscopy [133] In some instances the amount of sterol (e g , desmosterol <1 ng/ml) is undetectable in cerebrospinal fluid [134] even for selected ion monitoring TLC–GC

3 2 Phospholipids

This lipid class includes the phosphoglycerides and sphingolipids, all important membrane constituents The determination is particularly important in amniotic fluids, which deserves special mention

3 2 1 Phospholipids in amniotic fluids

Surface-active phospholipids have an important influence on the mechanical behaviour of the lungs. Alveoli may collapse in the absence of surfactant and this deficiency is responsible for the respiratory distress syndrome (RDS) Prediction of RDS is possible by analysis of phospholipids in the amniotic fluid As was quoted in a recent review [135], "at the present time it appears that the method of choice for determination of phospholipids in amniotic fluids is the TLC method". A large number of papers have appeared and continue to appear TLC and HPTLC of phospholipids and glycolipids in health and disease have been reviewed [136] Concentrations of lecithin (L), sphingomyelin (S) (the L/S ratio) and phosphatidylglycerol are the most important factors, as was evidenced by discriminant functional analysis [137] The L/S ratio increases from 0 9 (immature) to 2 6 (mature) between the 30th and 38th weeks of gestation. High concentrations of sphingomyelin increase the possibility of RDS. High concentrations of lecithin are correlated with high concentrations of phosphatidylglycerol, and it has been claimed that the presence of phosphatidylglycerol precludes the possibility of RDS [138] TLC assays of amniotic fluids from 346 patients have been evaluated [139] and the optimum diagnosis in neonates has been found to rely on phosphatidylglycerol and sphingomyelin concentrations plus the L/S ratio A ratio of 2 5 is a satisfactory cut-off value for distinguishing foetal lung maturity and immaturity [138].

Other methods have been proposed and compared with TLC For example, 180 amniotic fluids were checked using three methods two-dimensional TLC, a rapid slide agglutination test and an enzymic colorimetric procedure Apparently the immunological test is simple and rapid but relatively insensitive, as it detects phosphatidylglycerol at concentrations exceeding 2 μmol/l [140] Teng et al [141] performed the enzyme analysis of amniotic fluid phospholipids containing a choline group and compared the results with those obtained by TLC The time of assay is 10 min but only the L/S ratio is obtained. TLC development takes less than 25 min If ten samples are deposited on the plates within 10 min and a further 25 min are required for quantitation, an average of 6 min per sample is necessary for TLC Moreover, much more information is obtained on the plate with the further advantage of a preserved chromatogram Quantitative analysis of amniotic fluid phospholipids in diabetic pregnant women was carried out with HPLC, a foam stability index and TLC [142] and still favoured TLC A new test is based on fluorescence polarization spectroscopy, which measures the anisotropy of a fluorophore between the time it absorbs light and emits that light as fluorescence [143]. This method is much faster than TLC but no comparison of the reliability has been performed

Sample preparation A 5-ml sample of amniotic fluid is obtained by needle aspiration before rupture of the amniotic membrane The extraction of lipids is often carried out by the method of Gluck and Kulovich [144] or as slightly modified by Kolins et al [145] The procedure has been criticized as centrif-

ugation of amniotic fluid may alter the L/S ratio, acetone precipitation is not very effective in the separation of active lecithin from the other lecithin components A solid-phase extraction procedure in six steps has been proposed recently [135] and makes use of C_{18} extraction cartridges Aliquots of sample can be directly spotted [146]

Chromatography Bare silica gel is generally accepted as the most suitable sorbent The addition of ammonium sulphate to the adsorbent deactivates the layer and is effective on migration However, it has been claimed that dipping in 10% ammonium sulphate solution with subsequent activation at 100°C increases the separation, with the exception of phosphatidylethanolamine and phosphatidylserine [147]

Mixtures of chloroform, methanol and water were considered satisfactory as early as 1960 [148] Improved resolution of lecithin and sphingomyelin occurs with addition of either acid or base to the water Double development with slightly different mixtures [chloroform–methanol–acetic acid–water (70 30 4 3) and chloroform–methanol–acetic acid–ethanol–water (60 30 4 10 3)] is needed for the separation of phosphatidylcholine and sphingomyelin by circular HPTLC [149]

In two-dimensional planar chromatography the eluent may contain acetic acid in one direction and a base (ammonia, pyridine, triethylamine) in the other. This procedure takes advantage of the amphoteric characteristics of the solute as amine protonation occurs in one direction and phosphate or carboxyl dissociation in the other However, it has been claimed [150] that two solvent systems differing in ammonia will suffice to separate amniotic fluid phospholipids Comparison of both one- and two-dimensional TLC has been performed and the relative standard deviations (R S D) were comparable, with the exception of phosphatidylethanolamine, which exhibits a lower R S D in one-dimensional TLC [151] In a one-dimensional system a small amount of light petroleum increases the R_F between sphingomyelin and lecithin [152] In the two-dimensional system butanol or acetone can be added [153]

3 2 2 Phospholipids from other sources

Table 3 lists some proposed solvents for the separation of phospholipids on silica gel plates together with the solvents capable of separating lecithin and sphingomyelin for amniotic fluid analysis Four classes of solvents can be selected from the relevant literature Type I is a ternary mixture of chloroform, methanol and water The proportions are modified according to the purposes of the analysis Propanol (or isopropanol) may be added to tune the selectivity of type I solvents Type II solvents make use of ammonia (or another base, e g , tri- or diethylamine) Conversely, in type III solvents the base is replaced by formic or acetic acid Type IV solvents are mixtures of five solvents methyl acetate and aqueous potassium chloride are added to increase the selectivity (methyl acetate) and to form an ion-pairing system (potassium chloride)

However, the resolution of phosphatidylethanolamine from its oxidation products could not be achieved by TLC with either acidic, basic or neutral solvent systems [154]

The determination of phospholipids is important Alkenyl ether phospholipids (plasmalogen) have been described in membranes of animal and human cells A high plasmalogen content seems to be an important characteristic of electrically active tissues Phospholipids of many cancer tumours contain larger amounts of alkyl ethers than healthy tissues. Surfactant decreases the immune response of lymphocytes. Extremely high levels of ether phospholipids were found in the phospholipid composition of human eosinophils [155]. Preparation of the sample requires much care

Kolarovic and Fournier [156] compared five extraction methods for the isolation of phospholipids from biological sources and advocated the following method, which was checked on rat heart tissue The tissue was stabilized in 19 8 m*M* Tris–HCl–0 25 *M* sucrose–2 m*M* EDTA–0 1% bovine albumin (fatty acid free) buffer of pH 7 4 The mixture was centrifuged at 320 *g* for 10 min at 4°C and the supernatant liquid was further centrifuged at 3100 *g* for 10 min and at 105 000 *g* for 1 h The microsomal pellet was suspended in 7 9 m*M* sodium phosphate–0 397 m*M* EDTA–67 m*M* potassium chloride–0 1 *M* sucrose buffer of pH 7 45 A portion was mixed with water, warmed to 50°C and sonicated after the addition of hexane–propanol (30 20) The mixture was centrifuged at 2500 *g* for 1 min A portion of the supernatant liquid was separated by DEAE-Sephadex chromatography into acidic and non-acidic fractions (in the same way as in Section 3 3 1 for the preparation of gangliosides fractions) The two fractions were then submitted to TLC In the procedure of Helmy and Hack [157], interfering substances in the organic phase were extracted into butanol saturated with 0 85% sodium chloride solution Mitchell et al [158] developed extraction procedures for phospholipids and phosphoinositides in human erythrocytes, blood platelets and biological cells The lipid extracts of cells are treated, in the same way as above, with EDTA to chelate bivalent cations Subsequent use of type II and type III solvents in two-dimensional TLC successfully separates a large number of phospholipids.

In spite of the different claims, one may question whether centrifugation and sonication markedly change the chemical nature of the sample Touchstone et al [159] applied samples of cell suspensions directly to the preadsorbent zone of a concentration zone plate Extraction was carried out with three successive developments with chloroform–methanol mixtures This procedure extracts phospholipids from the sample and deposits them on the starting point

The selective affinity of boric acid for *cis*-diol compounds permits a high retention of phosphatidylinositol, which is well separated from the other phospholipids [160] The use of RP plates is not yet advocated for the separation of phospholipids from biological materials

TABLE 3

PLANAR CHROMATOGRAPHY OF PHOSPHOLIPIDS ON SILICA GEL PLATES

Sample	Solvent type[a]	Experimental procedure[b]	Detection	Solutes of interest	Ref
Amniotic fluid	I(a)	1D development then two separate 2D development with I(b) and III(c)	Periodic acid–Schiff stain densitometry	Phosphatidylglycerol	153
Cells, blood platelets, erythrocytes	II(a) III(a)	2D	Iodine vapour, scraping off, Subbarow reagent	Phosphoinositides	158
Amniotic fluid	III(b)	1D development, plates dipped in $(NH_4)_2SO_4$	Charring and densitometry	Phosphatidylethanolamine and phosphatidylserine not separated	147
Blood serum	I(a)	Sequentially with hexane–acetone (100 1)	Phosphomolybdic acid with $HClO_4$	All phospholipids	161
Pancrease, tissues	I(b)	2D for separation of all phospholipids	Schiff reagent	All phospholipids	157
Rat brain, tissues	I(c) III(c)	1D	Molybdenum blue	Selection of solvents according to the phospholipids to be separated	162
Whole brain, tissues, standards	III(d)	Chromarods	FID	Lipid classes and phospholipids	132
Biological fluids	III(e)	1D	Naphthol blue black, scraping off, extraction	Phosphatidylcholine and sphingomyelin	163
Cells from tissue cultures	IV	HPTLC	Staining with methanol–H_2SO_4–$MnCl_2$, fluorescence	Separation of six phospholipids	104
Body fluids	II(b), then III(c)	1D 2D	Acid fuchsin, uranyl nitrate	Phospholipid classes	164

Blood platelets	II(c)	Boric acid impregnated		Phospholipid classes	165
Amniotic fluid (in rat brain)	III(b) III(f)	U chamber, circular development	BBOT	Lecithin, sphingomyelin	149
Amniotic fluid	I(a), then $CHCl_3$, then hexane–$CHCl_3$ (30 10)	Successive developments		L/S ratio	95
Standards	II(d) and DEAE-silica gel			Eight major phospholipids	166
Amniotic fluid	II(e)	Two solvent systems differing in NH_3 content		L/S ratio	150
Standards	II(f), then III(c)	2D	Scraping off, $HClO_4$ digestion, NH_4 molybdate, 790 nm	Lipid classes	167
Standards	IV	1D	Comparison of detection reagents	L/S ratio	168
Standards	II(b), then THF–formaldehyde – CH_3OH– 2*M* NH_3 (10 7 2 1)	2D			169
Amniotic fluid	I(d)	Methanol-impregnated plates, then activation	Spraying with $MnCl_2$, fluorimetry	L/S ratio	170
Amniotic fluid	III(g)	Continuous development	Ammonium molybdate, λ=620 nm	L/S ratio	145
Amniotic fluid	II(g)	1D	P-specific spray	L/S ratio	171

[a]Solvent I = chloroform–methanol–water (a) 65 25 4, (b) modified with butanol, (c) 65 35 8, (d) 30 1 4 Solvent II = chloroform–methanol–ammonia–water (a) 48 40 5 7, (b) 70 30 5 2, (c) chloroform–ethanol–water–triethylamine (30 35 6 3 5), (d) chloroform–methanol–pyridine–ammonia–water (130 55 8 4 4), (e) chloroform–methanol–ammonia (5 4 1), (f) Chloroform–methanol–water–ammonia 65 35 3 2, (g) chloroform–ethanol–triethylamine–water (30 34 35 8) Solvent III = chloroform–methanol–acidic solution (a) chloroform–methanol–formic acid (55 25 5), (b) chloroform–methanol–acetic acid–water (55 16 5 2), (c) chloroform–acetone–methanol–acetic acid–water(10 3 2 2 1), (d) chloroform–methanol–acetic acid–water (45 15 15 0 2), (e) chloroform–methanol–acetic acid–water (110 55 16 6), (f) chloroform–methanol–acetic acid–ethanol–water (60 30 4 10 0 3), (g) chloroform–light petroleum–methanol–acetic acid (50 30 15 10) Solvent IV = chloroform–ethyl acetate–propanol–methanol–aqueous KCl (25 25 25 13 9)

[b]1D = one-dimensional, 2D = two-dimensional

3 2 3 Phospholipid detection

Many detection reagents have been proposed. Sherma and Bennett [168] compared many of these reagents and their ability to detect 2–3 μg amounts of solute

PMA, copper (II) acetate, copper (II) sulphate, Dittmer–Lester reagent [172] (prepared from molybdic anhydride and molybdenum in acidic solutions), bromothymol blue, 1,2-naphtoquinone-4-sulphonic acid, acid fuchsin–uranyl acetate, potassium *p*-toluidino-2-naphthalene-6-sulphonate, sulphuric acid in ethanol, nitric acid vapour and 2′,7′-dichlorofluorescein were examined with application to blood serum analysis All lipids and phospholipids with the exception of dipalmitoyllecithin could be detected within the range 100–200 μg on TLC plates and 50–100 μg on HPTLC plates These limits are similar to those on RP-18 plates No significant advantage of predipping was evidenced but the detection limits and reproducibility were enhanced when a predevelopment of the plate for cleaning purpose was done Copper (II) acetate works well on silica plates but less so on alkyl-bonded layers Eleven copper (II) compounds were checked by Bitman and Wood [173] and 10% copper sulphate in 8% orthophosphoric acid was selected as the best charring agent Subsequent scanning with a laser densitometer has been used for the determination of phospholipids in lymphocytes [174] Copper (II) acetate as a charring agent reacts only with unsaturated phospholipids whereas copper (II) sulphate reacts with both saturated and unsaturated phospholipids

Staining with the periodic acid Schiff stain according to Shaw is still in use [153] Formation of fluorescent derivatives with 8-anilino-1-naphthalenesulphonate gives a higher L/S ratio [175] Some published procedures are tedious in blood serum determination, zones are scraped, digested with perchloric acid and a colour reaction is produced with ammonium molybdate and malachite green or after staining with naphthol blue black, the plates are destained and the spots are removed and extracted with dimethylformamide prior to spectrophotometry [173] Molybdenum blue reagent is specific for phosphorus the plate is covered with an ammonium molybdate–phosphoric acid reagent for 1 min, dried for 5 min and densitometry is performed at 620 nm [145]

Fluorescent spots are formed by spraying or dipping the plates in manganese (II) chloride–sulphuric acid [176] Subsequent dipping in paraffin–hexane (10 20) doubles the fluorescence The use of 2,5-bis (5-*tert*-butyl-2′-benzoxazolyl) thiophene (BBOT) appears very efficient, the reagent is dissolved in the methanol part of the solvent system at a concentration of 20 mg/l and fluorescent phospholipid spots are stable for several hours [149] This reagent was tested in the determination of phospholipids in rat lung and 800 ng of sphingomyelin could easily be detected with a coefficient of variation of 0 3% Very simple and efficient detection of phospholipids makes use of film negatives with subsequent laser densitometry [177] Plates are immersed in a

buffer solution of *p*-toluidino-2-naphthalene-6-sulphonic acid in the dark and photographed Complex lipids are detected quantitatively at levels lower than 25 ng per sampling zone

An improved procedure for reproducible analysis with Chromarods and subsequent FID quantitation was published by Murray [178] Major phospholipids found in 3T3–L1 fibroblast and leukocyte membrane are quantitatively determined in the 1–30 μg range with an R S D of 0.2–6 0% Copper(II) sulphate-impregnated Chromarods have also been claimed to be reusable [179] The precision of the Iatroscan method was found to be comparable to that of GC for some phospholipids [180] Plasmalogen quantitation was achieved by Dembistky [181] using mild deacylation in a basic solvent followed by acid hydrolysis and subsequent determination of phosphorus after burning

Alvarez and co-workers [182–184] thoroughly investigated the hydrolysis of phospholipids with either acid, base or enzyme Plasmalogen identification in seminal fluids can be carried out with in situ hydrolysis to the vinyl ether lipids with trichloroacetic acid and subsequent detection with copper sulphate–orthophosphoric acid reagent Acid hydrolysis yields glycerol and aldehyde, the reaction is carried out on the plate and aldehyde is characterized. In situ alkaline hydrolysis produces glycerol and fatty acid derivatives, alkyl ether and alkenyl ether groups are not affected by this treatment Enzymatic hydrolysis with phospholipase A_2, C or D is performed directly on the silica layer and makes possible the characterization of the different phospholipids The detection limit is in the nanogram range which compares well with the 0.05 μg obtained by FID A quantitative determination of phosphatidylglycerol in amniotic fluid involves conversion of the phosphatidylglycerol into glycerol with phospholipase C and alkylphosphatase and subsequent determination of the glycerol formed [185] Enzymic generation of hydrogen peroxide from phospholipid followed by oxidative coupling of 4-aminoantipyrine to 2-hydroxy-3,5-dichlorosulphonate produces a red chromogen that is detected at 510 nm [186]

3 3 Gangliosides

Gangliosides are complex glycosphingolipids consisting of a hydrophobic acylsphingosine (ceramide) moiety to which hydrophilic oligosaccharide units containing sialic acid (N-acetylneuraminic acid) are attached In the Svennerholm nomenclature [187], a subscript M, D or T is used to indicate mono-, di- or trisialogangliosides, respectively The following nomenclature is used

G_{M1} = Galβ1–3GalNAcβ1–4(NeuNAcα2–3)Galβ1–4Glcβ1–1Cer,
G_{M2} = GalNAcβ1–4(NeuNAcα2–3)Galβ1–4Glcβ1–1Cer;
G_{M3} = NeuNAcα2–3Galβ1–4Glcβ1–1Cer,
G_{D3} = NeuNAcα2–8NeuNAcα2–3Galβ1–4Glcβ1–Cer,

G_{D1a} = NeuNAcα2-3Galβ1-3GalNAcβ1-4(NeuNAcα2-3)Galβ1-4Glcβ1-1Cer,

G_{D1b} = Galβ1-3GalNAcβ1-4(NeuNAcα2-8NeuNAcα2-3)Galβ1-4Glcβ1-1Cer,

G_{T1b} = NeuNAcα2-3Galβ1-3GalNAcβ1-4(NeuNAcα2-8NeuNAcα2-3)-Galβ1-4Glcβ1-1Cer,

where Gal = galactose, Glc = glucose, GalNAc = N-acetylgalactosamine, NeuNAc = N-acetylneuraminic acid and Cer = ceramide

The highest levels of gangliosides are found in the brain, where GM_1, GD_{1a}, GD_{1b} and GT predominate Tay Sachs disease is caused by a lack of N-acetylhexosaminidase and gangliosides accumulate in the brain In healthy individuals gangliosides constitute 6% of the lipids of the brain Increased serum or urinary levels of sialic acid or acetylated derivatives of neuraminic acid have been observed in inflammatory disease or cancers Gangliosides of human melanoma have been studied [188] and GM_3, GD_3, GM_2, GD_2 and alkali-labile gangliosides are most commonly expressed by these melanomas. However, the ganglioside distribution is heterogeneous and it is necessary to analyse the ganglioside composition of melanoma biopsies, before using monoclonal antibodies to melanoma-associated gangliosides for melanoma diagnosis

Ganglioside mapping can be used to check patients with San Filippo disease GM_3 is the major ganglioside in normal lymphoid tissue, increased contents of GD_3 were observed in the blast from children with T cell lymphoblastic malignancies [189] Four major gangliosides containing lactosamine or a lactosamine-repeating structure as the backbone core were recently isolated from human placenta with a combination of TLC, GC and proton magnetic resonance spectroscopy [190] The accumulation of gangliosides in cerebrospinal fluid of patients with gangliosidoses has been demonstrated The ganglioside composition of human milk is characterized by the predominance of GM_3 which, together with GD_3, constitutes $>95\%$ of the total gangliosides [191] GM_1 isolated from human milk was found in all fractions with enterotoxin inhibitory activity, whereas fractions without GM_1 were inactive [192]

3 3 1 Sample preparation

Ganglioside fractions are obtained by taking advantage of the amphipathic nature of glycolipids Total lipids are extracted with chloroform–methanol mixtures and gangliosides are then separated from this extract Quantitative extraction of gangliosides from brain has been thoroughly studied by Suzuki [193] When dealing with extraneural tissues or human erythrocytes with a low ganglioside content, the extraction procedure of Ledeen et al [194] is generally used This procedure, which has been slightly modified by Ando et al [195], involves extraction with chloroform–methanol (2 1) at 37°C for 30 min and re-extraction with ten volumes of chloroform–methanol (1 2) at 45°C for 30 min The extracts are mixed, adjusted to a chloroform–methanol–water

ratio of 30 60 8 (solvent A) and applied to a DEAE column which is eluted with 10 ml of solvent A and then 18 ml of chloroform–methanol–0 8 *M* sodium acetate (30 60 8) (solvent B) Neutral lipids and glycolipids are recovered in solvent A and effluent gangliosides and acidic lipids in solvent B, which is evaporated to dryness and the residue dissolved in 0 20 ml of 0 6 *M* methanolic sodium hydroxide and 0 4 ml of chloroform Warming at 37 °C for 1 h saponifies phospholipids. Neutralization with 0 03 ml of acetic acid followed by desalting on a Sephadex G-25 column eluted with chloroform–methanol–water (60 30 4 5) is then performed The eluate is evaporated to dryness and the residue is dissolved in 2 ml of chloroform–methanol (80 20) and applied to a silica column (previously washed with chloroform) and eluted with chloroform–methanol (80 20), which removes fatty acids and sulphatides Gangliosides are eluted with 15 ml of chloroform–methanol (2 3)

In the procedure advocated by Ando et al [195], mono-, di-, tri- and tetrasialogangliosides are sequentially eluted using a gradient from 0 to 0 6 *M* aqueous sodium acetate–chloroform–methanol (8 30 60) Salts are removed by gel chromatography using Toyo Pearl HW50 (Toyo Soda, Tokyo, Japan) Further purification is performed on an Iatrobeads 6RS-8060 (Iatron, Tokyo, Japan) column, and gangliosides are eluted with a linear gradient of chloroform–methanol–water from 83 16 0 5 to 20 80 5 [190] Another isolation purification procedure for gangliosides from plasma involves partitioning of the dried total lipid extract in diisopropyl ether–butanol–aqueous sodium chloride (60 40 50) and gel permeation Lipids from cerebrospinal fluid were extracted in chloroform–methanol, then applied to a silica PBA60 column for separation of phospholipids and gangliosides [196] Short-bed continuous development TLC was used to separate neutral glycolipids and gangliosides [197].

In the procedure of Pick et al. [198], total lipid extract is obtained at room temperature with chloroform–methanol through sonication Isolation of acidic and neutral lipid fractions was performed on DEAE-Sephadex A-25 in the acetate form For the recovery of acidic glycolipids the procedure was as follows after incubation the sample was dried in vacuo and the residue dissolved in cold HPLC-grade water, neutralized with dilute hydrochloric acid to pH 4 5 The salt concentration was adjusted to 0 1 *M* by adding water The sample solution was passed through an ODS cartridge at 1 5 ml/min, the acidic glycolipids being collected on the column After washing the cartridge with 50 ml of water the glycolipids were eluted with 30 ml of chloroform–methanol (10 20) Class fractionation was performed by OPLC by isocratic elution with chloroform–methanol (70 30)

3 3 2 Chromatography

Silica gel is an effective sorbent for separating the individual gangliosides The mobile phases used are chloroform–methanol–aqueous salts or ammonia, as previously described by Wherrett and Cuming [199] and Ando et al [200]

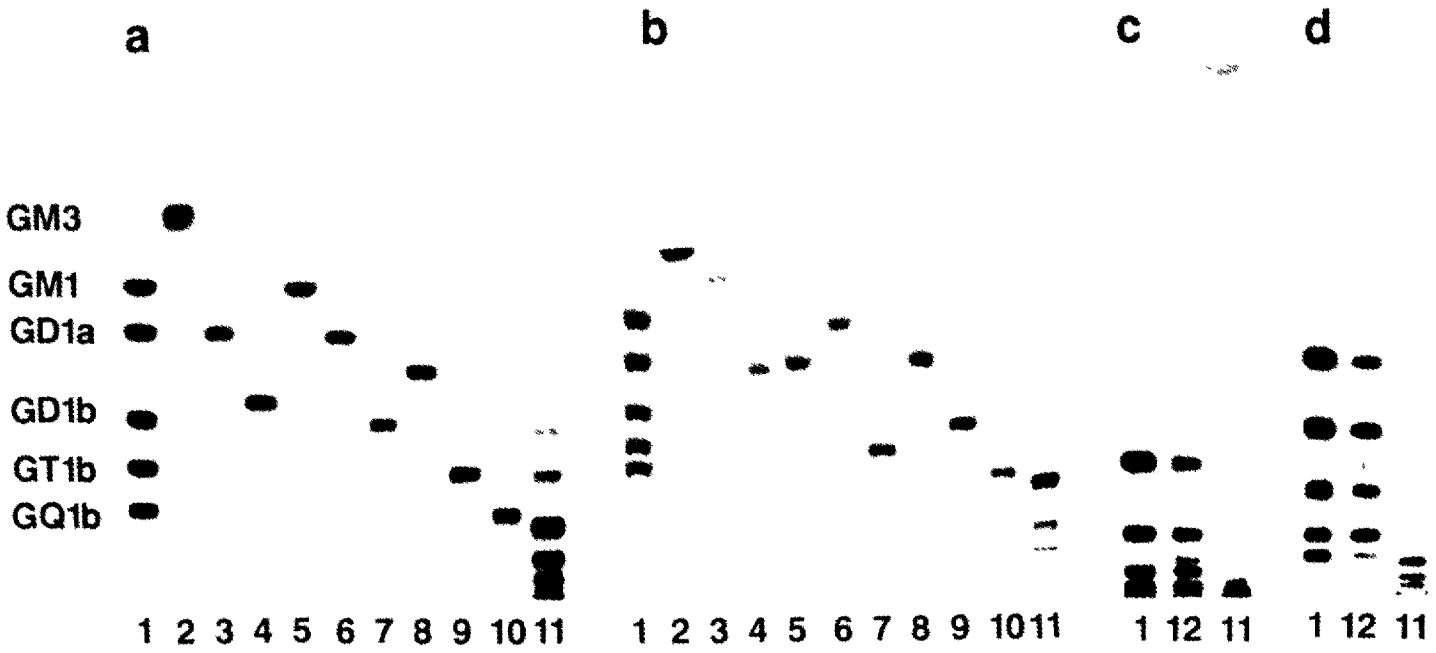


Fig 5 Ganglioside separations by HPTLC using the solvent systems (a) acetonitrile–isopropanol–50 m*M* aqueous potassium chloride (10 67 23, v/v/v), (b) acetonitrile–isopropanol–2 5 *M* aqueous ammonia (10 65 25) and (c) and (d) chloroform–methanol–0 2% aqueous calcium chloride (55 45 10) (c) and (40 40 11) (d) Samples 1 = a synthetic mixture of GM1, GD1a, GD1b, GT1b and GQ1b, 2 = GM3, 3 = GD3, 4 = GD2, 5 = GM1, 6 = GD1a, 7 = GD1b, 8 = GT1a, 9 = GT1b, 10 = GQ1b, 11 = codfish-brain total gangliosides, 12 = monkey-brain total gangliosides Each band of standard ganglioside contained 0 2–0 4 μg in sialic acid Gangliosides were located with resorcinol–hydrochloric acid reagent Reproduced from ref 195 with permission

or propanol–water–pyridine–ammonia–methyl acetate–0 2% potassium chloride [201] A much simpler eluent, tetrahydrofuran–50 m*M* potassium chloride, was claimed to give good separations [202] It was observed that the calcium sulphate binder (plaster of Paris) affects the resolution and including a salt such as calcium chloride or potassium chloride has been proposed for improving the separation [204] Halogen salts produce excellent separations whereas non-halogen-containing salts only slightly improve the resolution when compared with a system free from salts Strongly ionized cations are effectively associated with gangliosides The lower the polarity of a ganglioside, the faster it migrates on the silica layer and monosialogangliosides exhibit higher R_F values than di- and trisialogangliosides Within a group the mobility is affected by the number of hexose or hexosamine units Therefore, GM_3 migrates faster than GM_2 and GM_1

Chloroform–methanol–aqueous halogen salt systems are often quoted [194–196] and work well However, these solvent systems are very difficult to use in both HPLC and TLC and Ando et al [195] proposed a ternary mixture, acetonitrile–isopropanol–2 5 *M* aqueous potassium chloride solution (10 65 25) or acetonitrile–isopropanol–2 5 *M* aqueous ammonia (Fig 5) The order of mobility is $GM_3 > GM_1 > GD_{1a} > GT_{1a} > GD_{1b}$ and replacement of the aqueous salt by ammonia changes the sequence to $GM_3 > GD_{1a} > GT_{1a} > GM_1 > GD_{1b}$ Ammonia-containing solvents are obviously unsuitable for HPLC The change in selectivity has been previously observed and utilized in two-dimensional TLC Ohashi [206] used chloroform–methanol–aqueous ammonia in the first direction and *n*-propanol–aqueous ammonia in the second Chigomo et al [207] used chloroform–methanol–0 2% calcium chloride (50 40 10) and propanol–ammonia–water (60 20 10) in the orthogonal direction Membrane ganglioside changes in murine peritoneal macrophage and human promelocytic leukaemia were assessed by two-dimensional TLC [208]

As silica gel is capable of separating gangliosides on the basis of the oligosaccharide parts, it is expected that resolution on the basis of the ceramide apolar moiety can be carried out by RP-TLC RP-18 plates with acetonitrile–methanol–water (50 19 20) as the eluent permit the separation of a mixture of GM_1 molecular species containing C_{18} sphingosine, C_{18} sphinganine, C_{20} sphingosine and C_{20} sphinganine [209] Owing to the negative charge of sialic acid at pH 7 0, ion-pair chromatography is possible Tetrabutylammonium was unsuccessful but cetylpyridinium was satisfactory in the separation of GM_1, GD_{1a}, GD_{1b} and GT_{1b} [210]

Silica gel layers were only used in OPLC and a stepped gradient from chloroform–methanol (70 30) to chloroform–methanol–water (55 36 9) is able to separate GM_3, GM_2, GM_1, GD_{1a}, GD_{1b}, GT_{1b} [211] Optimization of the ternary mobile phase has been achieved with the Prisma model [212]

3 3 3 Detection

Picomole levels of gangliosides are readily detected, which makes layer chromatography very competitive Two procedures are in common use. spraying with a reagent and subsequent densitometry or immunostaining Spraying with resorcinol–hydrochloric acid reagent is very sensitive Subsequent heating at 140 °C yields blue spots for gangliosides Densitometry is carried out at 580 nm and calibration graphs are linear from 1 pmol (the minimum detectable amount) to 50 pmol [195] Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) is applicable with C_{18} layers [209]

3 4 Cerebrosides

Cerebrosides are neutral glycosphingolipids Glycosphingolipid nomenclature can be found in Wiegandt's book [213] Kidneys with Fabry's disease exhibit accumulation of galabiosylceramide (Galα1–4GalCer, Ga_2Cer) and globotriaosylceramide (Bg_3Cer) Separation of individual neutral glycosphingolipids is not well documented In a recent study, Ogawa et al [214] examined the separation of GlcCer, GalCer, Ga_2Cer, LacCer, Gb_3Cer and Gb_4Cer on silica gel plates with polar mixtures of solvents The most successful system was propanol–1 *M* ammonia solution–methyl acetate–water (75 5 5 25) but the neutral glycolipid composition of human kidney was best resolved with the proportions 75 10 5 15 GlcCer and GalCer were resolved without the need for the borate-impregnated silica layer advocated by Kean [215]

3 5 Sphingolipid detection with immunoassay

Sensitive immunoassay is capable of detecting trace amounts (0 1% of GM_1) of the total glycosphingolipids In the procedure of Towbin et al [203], the plate is sprayed with isopropanol–water (20 10) until visible and evenly wet and pressed in contact with a nitrocellulose sheet for 1 min to effect diffusion transfer ('blotting') The sheet is dried then rehydrated with pH 7 4 buffer solution and unconsumed binding sites are blocked by treatment with diluted horse serum Strips are cut and allowed to react with antiserum, then with a peroxidase-conjugated second antibody After washing, the glycosphingolipid bands are detected with hydrogen peroxide and 4-chloro-1-naphthol Human melanoma gangliosides separated by TLC are hydrolysed on the plate by *Vibrio cholerae* neuraminidase in 5 ml of buffer maintained on the plate for 18 h Products specific to gangliosides GM_2 and GD_2 are detected by an immunoperoxidase staining system after treatment with monoclonal antiganglio-tetraosylceramide [216]

In the procedure of Yamanaka et al [217], the plate is soaked in 10 m*M* sodium acetate buffer of pH 5 0 for 1 h and incubated with sialidase in the same buffer containing 0 25 mg/l sodium taurodeoxycholate The reaction is

stopped by washing the plate with 0 1 *M* phosphate buffer of pH 7 0 and the asialo compounds are detected by enzyme immunostaining According to Hirabayashi et al. [218], the plate is treated with *Arthrobacter ureafaciens* sialidase to remove all sialic acids, hydrolysis of gangliosides is performed with sodium taurodeoxycholate and the resulting asialo glycolipids GA_1 and GA_2 are reacted with affinity antipurified GA_2 and anti GA_1 and then with horseradish peroxidase-conjugated anti-rabbit immuno-γ-globulin The method is claimed to be highly sensitive (detection of picomole amounts) and reproducible

After treatment with a saturated solution of polyisobutyl methacrylate, nonspecific binding sites are blocked by incubation with buffered saline containing 1% of gelatin and 10% of preheated horse serum for 30 min Chromatograms are then incubated with a saline solution containing antibodies raised against gangliosides at 37°C for 2 h A second incubation is carried out with peroxidase-labelled immunoglobulin for 1 h at room temperature in the dark, detection is carried out with hydrogen peroxide–4-chloro-1-naphthol reagent Detection limits are 2–500 μg [205]

In the avidin–biotin enzyme system for immunostaining [219], the glycosphingolipids are separated on a silica gel layer, which is then coated with a thin layer of poly (isobutyl methacrylate) and immersed in phosphate-buffered saline containing bovine serum albumin. The plate is incubated with primary and then secondary biotinylated antibody for 30 min and the areas containing bound biotin are detected with use of biotinylated phosphatase avidin reagent The claimed sensitivity is similar to that obtained by RIA measurements Detection of picomole amounts of GD_{1b} and GT_{1b} is achieved by the direct binding of ^{125}I-labelled fragment of tetanus toxin.

Hansson et al [220] dipped the plate in 0 5% poly(isobutyl methacrylate) in diethyl ether After drying, the plate was immersed in 2% bovine serum albumin in phosphate-buffered (pH 7 3) saline solution containing sodium azide, then commercial antiserum against blood group antigen A or B (or both) was applied to the plate. Binding was detected with use of ^{125}I-labelled second antibody and autoradiography A similar method with antibodies directed against human milk sialyloligosaccharides was used to identify gangliosides from human meconium

Direct detection of the oligosaccharides of glycosphingolipids on TLC plates can be carried out by lectins conjugated to horseradish peroxidase and final detection by using a substrate medium consisting of hydrogen peroxide and 3,3′-diaminobenzidine [221] The plates were coated with poly (isobutyl methacrylate) and dipped in phosphate-buffered saline containing 2% bovine serum albumin and kept at room temperature for 1 h Lectin horseradish peroxidase conjugates were overlayed on the TLC plates following by 1-h incubation After thorough rinsing with phosphate buffer solution, the substrates were submitted to peroxidase substrate medium consisting of 3,3′-diaminobenzidine and

hydrogen peroxide The technique has been applied to gangliosides but some lectins are more specific for $\alpha(2\rightarrow6)$ than $\alpha(2\rightarrow3)$ linkages Combination of TLC with high-resolution MS and immunological reaction towards monoclonal anti-Forsseman antibody was used for the identification of pentaglycosylceramide in human kidney [222]

Identification of glycosphingolipids by FAB–MS [65] is tedious as extraction with methanol is followed by subsequent reactions, as described in Section 2 5

3 6 Porphyrins

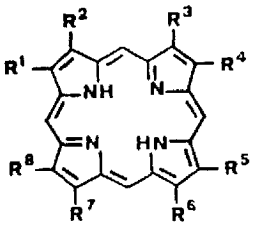
3 6 1 Importance of porphyrins

Porphyrins are tetrapyrrole derivatives with a porphine structure According to the different substituents, porphyrins are commonly named as indicated in Table 4.

Chelates are readily formed with metallic ions (Ni, V, Fe). Haemoglobin is formed by association of a protein (globin) and the iron–porphine chelate called haeme Haeme biosynthesis is caused by reaction of glycine with succinic acid and the last derivative is uropophyrin IX. Haematoporphyrin derivative is a

TABLE 4

FORMULAE OF COMMON PORPHYRINS



A = -CH_2COOH
E = -C_2H_5
M = -CH_3
P = -$(CH_2)_2COOH$

Name	Substituent and position							
	1	2	3	4	5	6	7	8
Uroporphyrin I	A	P	A	P	A	P	A	P
Uroporphyrin III	A	P	A	P	A	P	P	A
Coproporphyrin I	M	P	M	P	M	P	M	P
Coproporphyrin III	M	P	M	P	M	P	P	M
Protoporphyrin IX	M	V	M	V	M	P	M	P
Heptaporphyrin I	A	P	A	P	A	P	M	P
Hexaporphyrin I	M	P	A	P	A	P	M	P
Pentaporphyrin I	M	P	M	P	A	P	M	P
Mesoporphyrin IX	M	E	M	E	M	P	P	M

porphyrin mixture formed by treating haematoporphyrin dihydrochloride with 5% sulphuric acid in acetic acid Haematoporphyrin derivative has been studied by HPLC and it has been demonstrated that it is a complex mixture which may give irreproducible results in clinical tests [223]

Disorders arising from inherited or acquired defects in the biosynthetic pathway of haeme in bone marrow and liver are known as porphyrias [224] Porphyrias can be defined as errors in porphyrin biosynthesis. Porphyrin accumulation in tissues, blood, urine and faeces is the result of problems in haeme biosynthesis Porphyrinuria, which is the increased excretion of porphyrins in the urine, is a symptom, not a disease. In erythropoietic porphyrias, significant amounts are excreted in the urine together with large amounts (up to several thousand micrograms) in the erythrocytes. The skin symptom is photosensitivity with dermatoses, as in the case of cutanea tarda In protoporphyrias the amount of excreted porphyrin in urine is normal Hepatic porphyrias are either congenital or acquired (cirrhosis) Some porphyrins are, with respect to surrounding skin and muscle, preferentially taken up by tumour tissue Urinary and faecal porphyrin patterns are characteristic of each type of porphyria.

3 6 2 TLC of porphyrins

As was stated recently [225], TLC is the most widely used technique for the routine analysis of porphyrins Distinctive plasma porphyrin profiles are obtained even when total plasma porphyrin levels are slightly elevated Increasing numbers of papers advocate the use of HPLC, but spectrodensitometric scans of porphyrins indicate that some compounds are highly retained This would be troublesome in HPLC but not in TLC Porphyrins usually occur in the free forms, but esterified porphyrins are preferentially chromatographed Methyl esters of porphyrins are separated on silica gel with either binary or ternary mixtures of solvents as the mobile phase. Mixtures of hexane with methylene chloride, chloroform or ethyl acetate are considered to be satisfactory solvents Ternary mixtures advocated are benzene–ethyl acetate–methanol, toluene–ethyl acetate–methanol, methylene chloride–carbon tetrachloride–ethyl acetate and chloroform–kerosene–ethanol Separation is according to the number of carboxylic groups

The method published by Petryka and Watson [226] has been checked by Jork and Wimmer [227] Porphyrin extraction from urine or faeces is carried out by lipid precipitation from acetone and subsequent extraction with chloroform When urine is involved, the precipitation is performed with talc after addition of glacial acetic acid to pH 5 Methylation with methanol–boron trifluoride reagent is then performed, which is fairly rapid (20 min) and reliable Chloroform treatment is then necessary for the isolation of porphyrin methyl esters R_F values lie in the range 0.2–0 8 or 0 1–0 6, depending on the use of benzene or toluene in the developing solvent

Two-dimensional TLC was performed by Elder [228] in 1971 with carbon

tetrachloride–methylene chloride–methyl acetate–methyl isopropyl ether (20 20 10 10) in the first run and two successive developments with benzene–acetone (40 3) then chloroform–kerosene–methanol (200 100 7) in the orthogonal direction In spite of this tedious procedure, the spots are highly diagonalized and the resolution is not very high

The separation on OPLC could be optimized through the Prisma model and the selected solvent was hexane–ethyl acetate–diethyl ether–benzene–dioxane (50 30 10 6 4) [229]

Free carboxylic porphyrins can be separated with the ion-pairing technique on an alkyl-bonded layer with a mobile phase consisting of acetonitrile–N-cetyl-N,N,N-trimethylammonium bromide, 0 1 m*M* in aqueous acetate buffer (pH 4 1) [24] This system takes advantages of the ampholytic character of porphyrins as both their carboxylic acid group and ring nitrogen atoms ionize simultaneously so that the state of ionization and hydrophobicity are pH-dependent A simple ammonium acetate buffer corresponds to the isoelectric point of the porphyrins EDTA is added to the buffer to prevent the formation of metalloporphyrins Completely wettable RP plates are needed owing to the high water content In this mode, six free porphyrins (meso IK, copro I, penta I, hexa I, hepta I and uro I) are well separated Unfortunately no data on the applicability of the method to urine analysis were given A 30-min run time compares well with HPLC In recent HPLC studies by Saitoh et al [230], Garbo et al [231], Johnson et al [232] and Ho et al [233], porphyrin separation was readily carried out on an HPLC precolumn packed with 3-μm particles within an 8-min run time per sample, and the product purity was checked by TLC As an HPLC precolumn and TLC are similar in terms of plate efficiencies, such RP chromatography with buffered eluents should be tried in planar chromatography

3 6 3 Detection

Average values for faecal and urinary porphyrins in normal patients lie in the range 1–5 μg/g wet weight Porphyrin UV spectra exhibit absorption in the range 400–408 nm (Soret band) Porphyrins are brilliantly fluorescent in UV light [234] (Fig 6) with some differences in excitation and emission wavelengths On silica gel plates fluorescence is stabilized by treatment with dodecane–hexadecane There is no difficulty with detection and femtomole amounts of solutes are detectable on RP plates [24] with λ_{ex} 404 nm and λ_{em} 560 nm, the calibration graph is linear between 0 15 and 3 0 pmol

3 7 Prostaglandins

Prostaglandins (PGs) were isolated from the seminal vesicles of the sheep and are one of the most biologically active family of compounds ever discovered Eight groups of natural PGs are known They are unsaturated C_{20} fatty

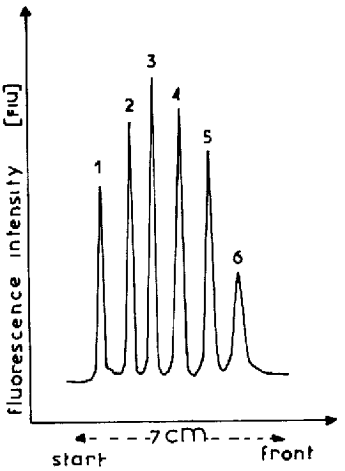
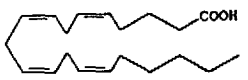


Fig 6 Fluorescence scan of a chromatogram track with 3-pmol amount of porphyrins Chromatographic conditions layer, HPTLC RP-18 WF254 s (10×10, 20×20 cm) (Merck), eluent, A = ethanol–dioxane (10 10, v/v), B = acetonitrile–isopropanol–acetate buffer, pH 4 1 (9 5 5, v/v/v), run time, A = 2 min, B = 22 min, migration distance, A = 15 mm, B = 70 mm Separation from start to front is mesoporphyrin IX (1), coproporphyrin I (2), pentaporphyrin I (3), hexaporphyrin I (4), heptaporphyrin I (5) and uroporphyrin I (6)

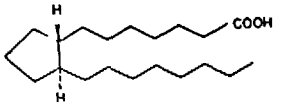
acids with a cyclopentane ring In addition to the letter designations to distinguish the substituted ring structure, the numerical subscript indicates the number of double bonds contained in the prostanoic acid chains Thromboxanes have a six-membered heterocyclic ring (Fig. 7) PGI_2 is characterized by a second heterocyclic ring and its degradation product is 6-keto-$PGF_{1\alpha}$

Quantitative analysis must fulfil severe requirements (a) high sensitivity as PGs occur in the nanogram or even picogram range in human tissues and body fluids; (b) great selectivity because the physico-chemical properties of the different PGs are very similar The quantitative measurement of PGs can be achieved by biological assays and particularly by RIA or chromatography HPLC and GC–MS after derivatization are often advocated but they are time-consuming TLC is simple and reliable, as indicated by recent studies on the cyclooxygenase products of arachidonic acid in the pulmonary system [235] or the determination of PGs in umbilical vessels of smoking and non-smoking mothers [236] The last report on PC appeared in 1978 [237] and the method is now considered obsolete owing to its poor sensitivity

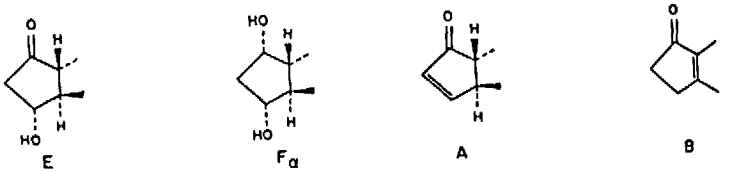
Silica gel is still widely used in TLC for the separation and identification of PGs Raajmakers [238] published the R_F values of 53 PGs and chloroform–methanol–acetic acid–water was considered to be suitable for the separation of all prostaglandin groups from pure substances Korte and Casey [239] utilized a preadsorbent TLC plate with a Celite area as preadsorbent With chloroform–isopropanol–ethanol–formic acid (45 50 0.5 0 3) they separated PGE_2

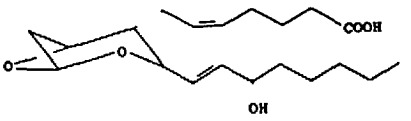


ARACHIDONIC ACID

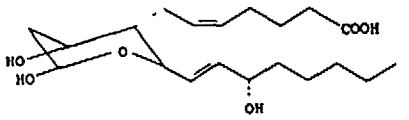


PROSTANOIC ACID





THROMBOXANE A_2



THROMBOXANE B_2

Fig 7 Structures of arachidonic acid, prostanoic acid, prostaglandins of the PG_1 and PG_2 series and thromboxanes A_2 and B_2

from $PGF_{2\alpha}$ and the major enzymatically formed metabolites of these PGs The order of decreasing R_F values is as follows arachidonic acid, 13,14-dihydro-15-keto-PGE_2, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, 15-keto-PGF_2, PGE_2, thromboxane B_2 and $PGF_{2\alpha}$. However 6-keto-PGF_1 and PGE_2 are not separated and chloroform–isopropanol–ethanol–formic acid (45 50 0.5 0.3) must

be used for this purpose This eluent was also proposed by Goswami and Kinsella [240] Smith et al [241] proposed a continuous development technique for the separation of 6-keto-PGF_1, PGF_2, PGE_2, 13,14-dihydro-15-keto-F_2, 13,14-dihydro-15-keto-E_2 and thromboxane B_2, the solvent system was ethyl acetate–acetone–acetic acid (90 5 1) In the rapid (1 h) miniature (on chromatogram sheets) TLC system for analysis of PGs and lipoxygenase products, Harris and Baenziger [242] proposed a slight modification with the use of ethyl acetate–isooctane–acetic acid–water in various proportions Bomalaski et al [243] examined a large number of mobile phases proposed in the literature and found that none could separate the major cyclooxygenase products of arachidonic acid from PGE_1 and PGE_2 (some eluents were mixtures of six solvents!) They advocated argentation chromatography with chloroform–methanol–acetic acid–water (90 7 5 5 0 8) Two-dimensional TLC has been proposed by Tsunamoto et al [244] for PGs and thromboxanes produced from ^{14}C-labelled arachidonic acid by a human neuroblastoma cell line The first development was carried out with ethyl acetate–isooctane–acetic acid–water (11 5 2 10) and orthogonal development with chloroform–methanol–acetic acid (90 8 6)

Using RP-TLC, Beneytout et al [245] separated PGF_2, PGE_1, PGE_2, PGA_1 and some arachidonic acid metabolites on silica gel modified with phenylmethylvinylchlorosilane The metabolites of ^{14}C-labelled arachidonic acid were separated on C_{18} plates with acetonitrile–water (70 30) [246] and eicosanoids on RP-18 with 0 0025 *M* orthophosphoric acid–acetonitrile (52 48) [247] Vasodilator PGE_2 analogues were separated either on silica gel G with chloroform–methanol–acetic acid (18 2 1) or on RP-12 plates with acetonitrile–water (35 65) [248]

To improve detection limits, 4-bromomethyl-7-methoxycoumarin derivatives have been proposed for some authentic samples [246] The optimum ratio of derivatizing agent to PG is 60 at 40°C within 15 min With these derivatives good linearity is observed in the range 0–200 μg This compares well with RIA and no cross-reaction can occur

Combination of TLC and HPLC has been advocated by Herman et al [249] They extracted PGEs from frog tissues and treated them with 0 5 *M* sodium hydroxide solution to convert PGEs into PGBs, which were purified by preparative TLC and subsequently analysed by HPLC on a C_{18} column with acetonitrile–water–1% acetic acid

Preparative TLC was used for the analysis of metabolites of arachidonic acid Zones containing the analyte of interest were removed and the extracts derivatized by silylation or methylation Subsequent determination was carried out by GC–MS [250] This procedure can be used for the quantitation of the lipoxygenase-derived conjugated 1,3-diene (12-hydroxyeicosatetraenoic acid) instead of a spectrophotometric procedure

3 8 Steroid hormones

3 8 1 Importance of steroids

Compounds with steroid structures occupy a key position in the human organism The level of the active hormones in serum and their elimination products in urine are of principal interest in the clinical diagnosis of hormone disturbances Glucocorticoid hormones contribute to adipogenic activity in human serum Addison's disease is glucocorticoid deficiency and Cushing's syndrome is glucocorticoid excess

In the female monthly cycle, various steroid hormones controlled by the hypophysis are mobilized during the different phases Maximum progesterone secretion occurs roughly on the 21st day of menstruation Progesterone will at the same time be metabolized and excreted in urine as pregnanediol glucuronate and *allo*-pregnanediol glucuronate During pregnancy estrogens are synthesized in large amounts by the placenta instead of ovaries In the early pregnancy test the pregnane-3α,20α-diol and *allo*-pregnane-3α,20α-diol which are present in urine in increased amounts can be readily detected by TLC

The most important steroids are divided into three classes C_{21}, C_{19} and C_{18} steroids

C_{21} steroids Progesterone the average content of the serum of menstruating women fluctuates between 0.25 and 3 5 μg per 100 ml Pregnanediol exceeds the normal value by about 2 mg/l ten days after conception, controlled when oral contraceptives are administered. Cortisol· less than 1% of total cortisol is excreted unchanged in urine. 6β-Hydroxycortisol· increased amounts are found in human urine during pregnancy and in cases of hyperadrenocorticism Prednisolone acetate· eliminated by healthy livers but not by patients with liver damage Aldosterone: normal aldosterone elimination in the 24-h urine is 10 μg

C_{19} steroids Testosterone· increased elimination in Cushing's syndrome (hyperactivity of the suprarenal cortex), increases in androgens are found when one or more enzymes are missing

C_{18} steroids Estriol, estradiol and estrone the ratio estriol/(estradiol+estrone) is normally unity for men and women, estrogenic steroid concentrations in human urine during pregnancy are important parameters for the gynaecologist

The chromatographic analysis of steroids is well documented, a book by Heftmann [251] appeared in 1976, the CRC Handbook of 1986 covers peptides, steroids and nucleic acids [252] and a review appeared in this Journal [253] on profiling steroid hormones

Many methods are available for steroid determinations· bioassay, spectrophotometry, GC–MS, RIA and HPLC with or without subsequent MS This explains the relative decline of the number of published papers on TLC However, GC requires hydrolysis, RIA is very sensitive but only a single compound

is detected and cross-reactivity with other steroids is possible According to Kabra [254], there are four types of methods for the determination of cortisol in plasma colour reactions, induced fluorescence, ligand assay and GC–MS or HPLC, but no mention was made of PLC, which has been extensively studied by Lewbart et al [255] and Funk et al [256] Steroid separations by PLC are easy and sample pretreatment or clean-up can be performed on the plate.

Determinations of urinary free cortisol by three methods, PC–RIA, HPLC–RIA and Sep-Pak chromatography–RIA, were compared [257] No marked differences in the results were found but several days are necessary for the first method and 3 h for the last

3 8 2 Chromatography

Major estrogens are easily separated Many NP systems have been published. Quantitation of estriol in pregnancy urine can be carried out on silica gel plates with butyl acetate–benzene (85 15) This mobile phase system was proposed in 1970 by Touchstone et al [258] and still advocated in a recent book [259]

As the separation of estrogens is very easy, the urine can be treated with β-glucuronidase and free estrogens are separated by TLC Benzene–acetone (90 10) [260] or chloroform–ethyl acetate (80 20) at 32–72% humidity are claimed to be efficient for separating major estrogens and androgens [261] A good two-dimensional thin-layer chromatogram was published by Taylor [262] as early as 1972, using chloroform–methanol–water (94 6 0 5) in the first direction followed by cyclohexane–ethyl acetate (50 50) in the second In a similar procedure, C_{19} steroids were separated with 1,2-dichloroethane–methyl acetate (80 20) followed by hexane–hexanol (65 35) [263]

Pregnanediol and pregnanetriol in urine extracts are well separated by TLC with silica gel plates and chloroform–acetone (80 10) or chloroform–acetone–methanol (75 15 10) [264] Two solvents and four successive developments are possible [265]

Cellulose layers [266] or polyamide impregnated with propylene glycol [267,268] resolve the estrogens according to their liquid–liquid distribution coefficients, but the use of cellulose plates for steroid separations is not very popular

The relationship between retention and hydrophobic character of steroids is well documented (see, e g , refs 269 and 270), and the availability of alkyl-bonded plates has given rise to new experimental procedures in the PLC of steroids The order of retention is highly correlated with the octanol–water partition coefficient and the retentions of estrogens, androgens, gestogens and corticoids were studied with methanol–water mixtures on RP-12 [271] and phenyl-bonded plates [272]

A perfect resolution of estrone sulphate, estradiol β-glucuronide, estrone β-glucuronide, estriol 16α-glucuronide, estriol 3α-glucuronide and estriol 3-sul-

phate was observed on RP-18 layers with the ion-pairing mobile phase methanol–0 5% tetramethylammonium bromide solution in water (45 55 or 50 50) [273].

Prechromatographic derivatization by formylation of the phenolic group and conversion of the resulting aldehyde into a fluorescent derivative with 1,2-diamino-4,5-dimethoxybenzene and subsequent separation on an RP layer or HPLC with methanol–aqueous phosphate buffer looks more tedious [268].

Very promising are cyano-bonded plates; estriol, estradiol and estrone are very easily separated with light petroleum–acetone (80 20) and 1,4-androstene-3,17-dione, progesterone and pregnenolone with light petroleum–ethanol (80 20) [274]. Two-dimensional chromatography can be carried out with an NP-like solvent (light petroleum–acetone) in the first dimension and an RP-type solvent (acetone–water) in the orthogonal direction

Diol-bonded silica plates exhibit very similar behaviour and heptane–ethyl acetate or heptane–isopropanol permit good separations of major estrogens and androgens [275]. An immobilized 3,6-dioxaoctanedioic amide substrate has been claimed [276] to be very selective in HPLC, but no attempt at PLC has been made.

With OPLC, twenty major steroids were separated in a two-dimensional system on a silica gel layer with light petroleum–water–saturated ethyl acetate (90 10 1) or chloroform–phenol (90 10) [277]

3 8 3 Detection

Coupling of estrogens with azo dyes has provided a simple means for the preparation of colour derivatives which may be separated by TLC and then subjected to densitometry Coupling of estrogens with Fast Violet Salt B to produce a yellow derivative was used by Lewbart et al. [255] for the quantitation of estriol in pregnancy urine. Prechromatographic derivatization of plasma cortisol with dansylhydrazine is performed in the dark [256] and HPTLC plates are examined by in situ fluorescence of the derivatives (λ_{ex} 365 nm, λ_{em} 460 nm), with dansyl chloride the detection limit is 1 ng [278] Shackleton [253] used three different plates with three different reagents for selective detection *m*-dinitrobenzene for seventeen oxosteroids, Blue Tetrazolium for α-ketols and antimony trichloride for pregnanetriol

Spraying with simple reagents such as rubeanic acid [279], which is a general spray reagent for steroids, yields excellent results Other recommended reagents are manganese(II) chloride with subsequent heating at 120°C and scanning at 366 nm [274] or charring of ammonium sulphate-preimpregnated plates after a *p*-toluenesulphonic acid spray [280] Spraying with 0 1 m*M* 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in toluene–acetic acid (70 30) and densitometry [281] has been claimed to be very sensitive for estriol 16β-glucuronide Calibration graphs are linear from 0 2 to 2 0 nmol Quantification of ppb levels of estrogen glucuronide in urine was achieved by spraying with 10%

sulphuric acid in ethanol, subsequent heating and fluorescence detection (λ_{ex} 366 nm, λ_{em} 509 nm) [282]

17-Ketosteroids and metabolites were detected in urine by TLC with videodensitometry [281] The R.S D is high (23%) but nevertheless the authors claimed that rapid information is given on endocrine diseases.

Enzyme colour development of urinary 3-hydroxy steroids on a TLC plate was performed by Yamaguchi [284,285] for excretion patterns. The enzymic detection is effected as follows

$$\text{substrate} \xrightarrow{\text{enzyme}} \text{product} \xrightarrow{\text{catalyst}} \text{formation of dye}$$

Hydroxysteroids are converted into oxosteroids by hydroxysteroid dehydrogenase reactions·

$$3\alpha\text{-hydroxysteroids} + NAD^+ \rightarrow 3\text{-oxosteroids} + NADH$$

$$3\beta,17\beta\text{-hydroxysteroids} + NAD^+ \rightarrow 3,17\text{-oxosteroids}$$

$$NADH + INT \xrightarrow{\text{diaphorase}} \text{formazan}$$

When 3β-hydroxysteroid oxidase reaction is involved, hydrogen peroxide is formed, which reacts with phenol and 4-aminoantipyrine:

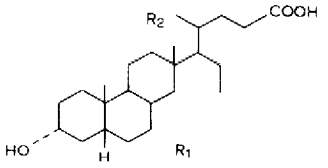
$$H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye}$$

In the same way, an enzymic assay of urinary estriol is performed prior to separation by TLC for the determination of urinary testosterone, epitestosterone and androstenedione [286].

3 8 4 Combined methods

Analysis of testosterone conjugates in urine and serum was carried out by TLC separation of sulphates and glucuronides and subsequent RIA [287] Combination with spectrophotometry [288] is fairly simple. Curtius and Muller [289] achieved a GC–TLC separation of a hormone mixture. The first dimension involves a GC separation, 10% of the effluent being directed to FID and the remainder spotted on a TLC plate Combined TLC–MS without substance elution was used in the direct identification of steroids [290]

3 9 Bile acids



	R_1	R_2
Lithocholic	H	H
Deoxycholic	H	OH
Chenodeoxycholic	OH	H
Cholic	OH	OH

3 9 1 Planar chromatographic separation

The bile acids which occur in the free state or as the more polar taurine and

glycine conjugates possess oxygen-containing functional groups in positions 3, 6, 7, 12, 16 or 23 (α- or β-isomers), in addition to the cholane C_{24} skeleton Bile [291], serum [292,293], duodenal contents and crude faecal extracts [294] are examined for the presence and quantitation of bile acids Separation of conjugated and unconjugated bile acids from biological fluids can be carried out by double development on silica gel with propionic acid–isoamyl acetate–propanol–water (30 40 20 10) for 10 cm followed by isooctane–diisopropyl ether–acetic acid–isopropanol (20 10 10 10) to the top of the plate [295] Chloroform–ethyl acetate–acetic acid–2-methoxyethanol (9 9 2 1) was advocated by Szepesi et al. [296] for the isolation and determination of chenodeoxycholic acid and related compounds Cholic, chenodeoxycholic and lithocholic acids and their 3-keto derivatives are separated on silica gel with hexane–methyl ethyl ketone–acetic acid (56 36 8) and diethyl ether–acetic acid (99 1) [297] R_F values of 28 bile acid standards were reported [298], together with colours produced after spraying with *p*-hydroxybenzaldehyde–sulphuric acid and heating. Analysis of individual free bile acids in serum involves protein separation, alkaline hydrolysis, solvolysis of conjugated bile acids and purification of lipids prior to separation by TLC on a silica gel plate with a ternary mixture of solvents [heptane–ethyl acetate–acetic acid (5 5 7)] [299] In this mode cholic, chenodeoxycholic and lithocholic acids are well separated (R_F values 0 41, 0 47 and 0.72, respectively) but not information is available on the amount of conjugated bile acids. A 500-μl sample from newborns and children is treated with ion-exchange resin prior to TLC separation [292] Depending on the sample pretreatment, 15% of phospholipids may co-migrate with bile acids and extraction of bile acids by liquid–liquid partition between methanol and chloroform can eliminate this interference [300]

The use of alkyl-bonded silica is increasing In one of the first application notes on RP-2 plates from Merck [301], the separation of cholesterol and bile acid derivatives with methanol–acetic acid (80 20) as developing solvent was reported A ternary mixture [ethanol–0 3% calcium chloride–dimethyl sulphoxide (50 50 4)] was proposed by Levitt and Touchstone [291] for the separation of bile acids from rat bile It must be pointed out that this is one of the very rare papers on the use of dimethylsulphoxide as a solvent. Derivatization to methyl esters and chromatographic mobilities of 26 theoretically possible hydroxylated derivatives substituted in one or more of the C_3, C_7 and C_{12} positions of methyl 5β-cholanate were thoroughly studied by Iida et al [302] RP plates are effective for the separation of the di- and trihydroxy stereoisomers, whereas the less polar monosubstituted isomers were well resolved in an NP system Combination of both would be very efficient A promising stationary phase is cyano-bonded silica, on which Okamoto et al. [303] performed some separations of cholic acids Bile salts are easily separated from bilirubin on Chromarods [107]

3 9 2 Detection

Fluorimetry is the most sensitive detection technique, as 0 05–0 1 μg of free bile acids and bile acids conjugated with glycine or taurine are detected by in situ fluorimetry [292,304] Bile acids are converted into fluorescent derivatives by reaction with 5% perchloric acid in methanol [305]. Spraying with 10% sulphuric acid and subsequent heating yields fluorescent, light blue spots on a dark violet background, detection at 370 nm is reliable with a linear calibration graph from 30 to 240 ng [303] Elution from the plate and the use of an enzymic fluorimetric method were proposed by Jagarinec and Parag [299], but the coefficients of variation are high ($>20\%$) for deoxycholic and lithocholic acid

Quantification of total bile salts (sodium salts of bile acids) can be carried out on Chromarods with an Iatroscan [107] The calibration graph is linear from 0 5 to 8 μg

A comparison between GC and PLC has been performed and, with the exception of deoxycholic acid, correlations between the two methods were highly significant [291]

3 10 Purines, pyrimidines, derivatives of nucleic acids

The analytical separation of nucleobases, nucleotides, nucleosides, purines and pyrimidines and related compounds is a major task in biochemistry Routine PLC is not in widespread use and the number of papers related to this topic is slightly declining

Owing to the possible protonation of these 'flat' bases, ion exchange looks convenient Uridine, uracil, uridine 5′-monophosphate, uridine 5′-diphosphate, uridine 5′-triphosphate and cytosine were separated on polyethyleneimine–cellulose with 0 55 *M* lithium chloride–0 2% formic acid at 0 °C [306] This support has been used to elucidate the structures of oligodeoxyribonucleotides by two-dimensional TLC [307] Organic ion-exchange resins have been used by Tomasz [308] and Elodi and Karsai [23] The layer in H^+ or NH_4^+ form is more convenient and in deionized water the order of decreasing migration rate is adenine, cytosine, guanine and uracil No recent report has appeared on this type of chromatographic support

As was mentioned above, amino-bonded silica gel is a weakly basic ion exchanger Jost and Hauck [309] observed that the four dinucleoside diphosphates, nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD) and their reduced forms (NADPH and NADH), are separated in this sequence on the basis of charge differences

Coating of cellulose with alcohol–water mixtures has been used for screening immune deficiency due to adenosine deaminase and purine nucleoside phosphorylase deficiency [310] A 1-μl amount of urine is necessary, deoxyadenosine and deoxyguanosine are separated from other urine constituents with

aqueous sodium acetate (3%) lithium chloride solution–methanol (30 70) With aqueous solutions of ammonium sulphate as the mobile phase a linear decrease in the R_F values of nucleosides at higher salt concentrations was observed [311].

The separation of adenine, adenosine, inosine, hypoxanthine, 2-adenosine 5′-monophosphate, 5-adenosine 5′-monophosphate, adenosine 5′-diphosphate, adenosine 5′-triphosphate and dibutyryladenosine 5′-monophosphate has been performed on silica gel plates [312] Two-dimensional separation of adenine, adenosine, hypoxanthine, xanthine, uric acid and inosine is possible on silica gel plates with *n*-butanol–ethyl acetate–methanol–ammonia (11 3 3 3) followed by *n*-butanol–methanol–ammonia (4 3 3) [313] In the detection of purines the proportions of these mixtures are slightly changed [314] TLC on silica gel was preferred to TLC on cellulose or paper electrophoresis for the analysis of the products of DNA modification by methylases [315]

Alkyl-bonded layers are by far the most efficient. Cadet et al [316] separated radioinduced degradation products of 2′-deoxyguanosine The two pyran anomers resulting from imidazole ring opening exhibit high R_F values owing to their less aromatic character Successive developments with methanol–water (10 90) resolved N-6-(deoxy-β-erythropentapyranosyl)-2,6-diamino-5-hydroxy-5-formamidopyrimidine from the α-anomer The resolution of the α- and β-anomeric forms of adenosine and deoxyadenosine has been achieved on a chiral plate with methanol–water–increasing amount of acetonitrile (50 50 30→50 50 400) [317] On these plates β-nucleosides had higher R_F values than the α-nucleosides and thymidine does not react with copper(II)

A very useful paper by Lepri et al [318] appeared recently They examined the PLC mobilities of 58 purine and pyrimidine derivatives on an alkyl C_{12}-bonded layer with either a salt-containing aqueous mobile phase or a non-aqueous solvent The R_F values are related to hydrophobic moiety, acid–base constants and association of the species in solution With C_{18} alkyl-bonded silica and an eluent containing dodecylbenzenesulphonic acid, ion pairing occurs and different R_F values are observed This might be of great importance for possible two-dimensional separations Tables of R_F values for every system are given in the paper

Visual detection of 0 01-μg amounts by spraying with aqueous 1% uranyl acetate is claimed to be possible [314] TLC associated with radiochromatography permits the quantitation of as little as 74 pmol of adenosine and TLC associated with liquid scintillation counting 410 pmol of adenosine [312] In the procedure of Maddocks et al [310] for screening adenosine deaminase deficiency, cooling in liquid nitrogen is carried out and phosphorescence of bands is recorded on X-ray film

In the diagnosis of adenine phosphoribosyl transferase deficiency, 1μl of urine is submitted to TLC and adenine is detected by its blue phosphorescence at liquid nitrogen temperature in the same way as above [319] Videodensi-

tometry as designed by Pongor [73] has been extensively used by Elodi and Karsai [23], who claimed an error reproducibility of less than 1%

Patients with inherited adenyl succinase deficiency excrete large amounts of succinyladenosine and succinylaminoimidazole carboxamide riboside Pretreatment of urine samples was performed by ion exchange and two-dimensional TLC on cellulose plates was then carried out The solutes of interest were detected via the dull blue colour produced after spraying with Pauly reagent Concentrations were determined by HPLC [320]

Uric acid is excreted as the principal end-product of purine metabolism Normal serum levels range from 25 to 75 μg/l It is not usually analysed by TLC, an improved reagent is capable of detecting 5–50 μg of uric acid but was tested only on bird excreta [321] Conversion of hypoxanthine into uric acid by xanthine oxidase was the last step in a micro-determination of purine nucleoside phosphorylase activity in peripheral blood lymphocytes with a Cobas Bio analyzer The results were compared with those given by TLC and radioautography [322]

3 11 Urinary organic acids

These are the end-products of the intermediary metabolism of carbohydrates, amino-acids, lipids and nucleotides In this field a GC–MS data system such as the MSSMET has been proved very useful Prior to GC–MS analysis a TLC purification step is often required, as in the investigation of organic acids in samples from patients with diabetes mellitus, where four fractions were separated on silica gel plates prior to GC and GC–MS [323]

Homovanillic acid (HVA) and vanillylmandelic acid (VMA) are acidic metabolites of catecholamines HPLC with electrochemical detection gives excellent results However, a quantitative TLC–spectroscopic method of quantitation was proposed by Ufer-Weiss et al [324]. Chloroform–ethanol–formic acid (50 10 10) separates HVA and a second development with different proportions (50 20 2) resolves VMA and 3,4-dihydroxymandelic acid (DMA) The plates are dipped in ethylenediamine–chloroform (25 75) after addition of 5% dimethylsulphoxide and fluorescence scanning at 390 nm is performed The minimum concentration detected is 2 5 μg with a claimed reproducibility of 90% In a study of 200 urine samples from patients with neuroblastoma, Tuchman et al [325] suggested qualitative analysis by TLC and quantitative analysis by GC

Determination of methylmalonic acid in plasma, urine and liver can be carried out by TLC on cellulose with *n*-butanol–acetic acid–water (13 3 5) and detection with Fast Blue B [326] or diazotization followed by densitometric scanning of a photocopy of the chromatogram

Alumina- or cellulose-coated plates were suggested by Kuroda et al [327] for the analysis of 5-hydroxyindole-3-ylacetic acid and indol-3-ylacetic acid,

with subsequent detection and quantification by phosphorescence. Calibration graphs were linear from 10 pmol to 250 nmol.

In our opinion, the detection of these acids by TLC is easy but quantification by HPLC is more reliable. Combination of TLC with either GC or HPLC is obviously the best choice

3 12. Carbohydrates

Numerous diseases are accompanied by increased elimination of various sugars in the faeces and urine. In diabetes mellitus, an increase in the amount of sugars other than glucose is observed and these sugars are thought to be involved in the progress of microangiopathy Separation of sugars in the free state is tedious. All chromatographic techniques can be used Standard mixtures are generally well resolved and good HPLC traces on an anion-exchange resin (calcium or lead type) or on amino-bonded silica can be found in a extensive literature. PC and TLC have been used extensively but they are now considered to be inferior methods for carbohydrates In the review by Robards and Whitelaw [328] it is stated that in the period 1980–1985 TLC represented 14% of the published papers and PC 3%. Stationary phases utilized in PLC are silica gel with binary, ternary or quaternary mixtures of solvents such as butanol (water-saturated)–triethylamine, ethyl acetate–methanol–acetic acid–water, propanol–methanol–water and propanol–water [329]. The mobility of sugars on silica gel depends on the molecular mass and the number of hydroxyl groups. Plates of silica gel of very large pore size (Si 50 000 from Merck) have been advocated [330] with ammonia-containing eluents. Samples of 1 μl of urine from patients with melituras were used for two-dimensional TLC on cellulose plates [331]. Use of a copper(II)-loaded stationary phase has been proposed [332] for separating mixtures of carbohydrates Water-containing solvents or pure water are used for development.

In biomedicine, PLC is the method of choice when a single carbohydrate is looked for. Lactulose is checked in the urine of patients with cystic fibrosis using silica gel with a propanol–borate buffer solvent [333]. Sucrose in biological samples was separated on silica gel with ethyl acetate–isopropanol–water (60 30 10) whereas the mobile phase for fructose was isopropanol–*n*-butanol–0 5% aqueous boric acid (50 30 20) followed by *n*-butanol–acetone–0.5% boric acid (40 50 10) [334]

The most important application is the detection of the zone of interest and possible quantification Patzsch et al. [335] compared some monosaccharide separations on silica plate, amino and Si 50 000 plates with subsequent post-chromatographic derivatization with aniline, diphenylamine, phosphoric acid, 4-aminohippuric acid, anilinephosphoric acid, 2-aminophenol, anthrone, urea, naphthoresorcinol, dimedone, PMA, vanillin and zinc chloride. The limit of detection could be lowered to 5–30 ng per spot. Derivatization with dabsylhy-

drazine and TLC of monosaccharide dabsylhydrazones on silica gel with a ternary solvent (e.g , acetonitrile–*n*-butanol–ethyl acetate, 15 15 1) permit the detection of picomole amounts of sugars [336] This compares well with HPLC detection sensitivity as it is claimed that nanomole amounts are very easily detected and picomole detection requires a pulse amperometric detector

Diagnosis of galactosaemia was carried out by impregnation of a filter paper with blood followed by extraction with 60% isopropanol and TLC separation on silica gel with propanol–methanol–water (40 20 7 7) spraying with *o*-aminobenzenesulphonic acid and quantification by scanning densitometry [337]

In lysosomal acid deficiency (Pompe's disease) diagnosis, urine was desalted on ion-exchange resins, the eluates were evaporated to dryness and the residues dissolved in water and the tetrasaccharide band was located on the TLC trace. Lactobionic acid can interfere [338] Oligosaccharides released from gangliosides by endoglycoceramidase were purified on Sep-Pak C_{18} and a Sephadex column TLC was then carried out on polyamide with acetonitrile–phosphate buffer solution (1 1 to 2 1) and the oligosaccharides were immobilized on the layer by reaction with $NaBH_3CN$ and submitted to immunostaining and densitometry [339].

OPLC greatly improves the separation. Vajda and Pick [340] separated mono-, di- and trisaccharides on a silica HPTLC plate with elution with acetonitrile–aqueous ammonia–aqueous potassium chloride They used OPLC in the TLC mode with ethanolic orcinol–sulphuric acid detection

3 13 Amino acids

Chromatographic separation of amino acids is very well documented and an excellent paper on the topic appeared in this Journal [341] HPLC is widely used and *o*-phthalaldehyde derivatization has proved suitable for either fluorescence or electrochemical detection PC is still in use as dabsyl derivatives are very easily detected [342] and simple screening of abnormalities in amino acid excretion is quick and inexpensive [343]

Comparison of layers for amino acid separations was performed by Sleckman and Sherma [344,345] Cellulose, silica gel, ion-exchange and alkyl-bonded stationary phases were evaluated and tables of R_F values for essential and non-essential amino acids were given TLC on RP layers has no advantages over adsorption, partition or ion exchange Owing to the aqueous nature of the mobile phases, the development times are long (2–11 h) The best results were obtained on Fixion layers, 0 12 mm thick, 1–20 μl of untreated urine can be directly spotted but 6 5 h are needed for completion of the chromatographic run

Ion-exchange TLC was also advocated by Issaq and Devenyi [346] Application to screening of blood and urine of newborns was carried out by Kovacs and Kiss [347] Detection of phenylketonuria was performed by liquid–liquid

extraction of the urine on Sep-Pak cartridges, elution of phenylalanine and keto acids with methanol and ion-exchange TLC on Fixion plates [348]

Selectivity and consequently separation can be improved by mixing sorbents as proposed by Gullner et al [349] or by impregnation of RP plates with dodecylbenzenesulphonic acid [350] For this purpose two-dimensional TLC of dansylamino acids, as advocated in 1980 by Macek et al. [351], is still the best system An example is the amino acid analysis of patients with leucinosis, citrullinuria and phenylketonuria. The method does not require deionized urine or deproteinized serum samples [352,353] and can be combined with autoradiography to detect 0.1 pmol of amino acids [354].

Urinary methylhistidine isomers are separated and determined with methanol and chloroform–methanol–ethyl acetate–triethylamine (70 10 10 5) [355] Screening of aspartylglycosaminuria is performed in this way [356]. TLC is very sensitive, as was exemplified in the screening and diagnosis of heterozygous cystinuria; the highest sensitivity of TLC compared with spectrophotometric tests has been demonstrated [357] Detection of 400 fmol of

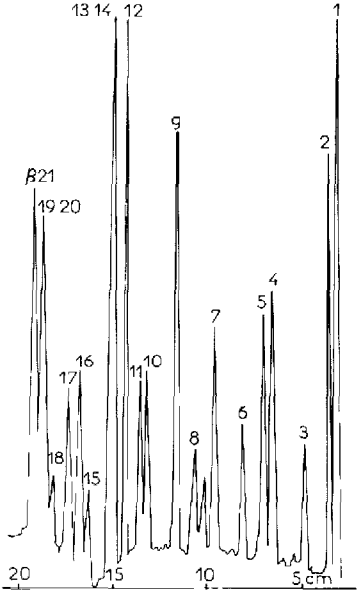


Fig 8 Separation of PTH-amino acids by OPLC Chromatographic conditions layer, HPTLC silica gel 60 F_{254} (20×30 cm, experimental plate, Merck), 1st eluent, chloroform–methanol–acetic acid (90 10 3, v/v/v), 2nd eluent, dichloromethane–ethyl acetate (90 10, v/v), 1st development time, 20 min at 16 cm, 2nd development time, 34 min (continuous development) Peaks 1 = $CySO_3K$, 2 = His, 3 = DMSO, 4 = Asn, 5 = Gln, 6 = Asp, 7 = Ser, 8 = Glu, 9 = Thr, 10 = Lys, 11 = Tyr, 12 = Gly, 13 = Trp, 14 = Ala, 15 = Met, 16 = Phe, 17 = Val, 18 = Nle, 19 = Ile, 20 = Leu, 21 = Pro

fluorescent Lissamine Rhodamine B sulphonyl aryl derivatives of amino acids can be performed [358]

The application of layer liquid chromatographic techniques is popular for the analysis of phenylthiohydantoin (PTH)-amino acids. A recent review appeared on the TLC of PTHs of amino acids on different stationary phases [359] The separation of all common PTH-amino acids is better achieved by HPLC Schuette and Poole [360] used continuous multiple development with five development steps and four changes of mobile phase composition to separate eleven derivatives The situation is more favourable in OPLC Fater and Mincsovics [361] used chloroform–methanol–acetic acid (90 10 2) for the resolution of polar PTH-amino acids and dichloromethane–ethyl acetate (90 10) for the resolution of non-polar PTH-amino acids with an increased migration distance (Fig. 8).

4 APPLICATION TO EXOGENOUS SUBSTANCES

The planar chromatography of pharmaceuticals and drugs would deserve a separate review. PLC is extensively used in this area and the number of references listed in the annual reviews in Analytical Chemistry is the largest of all the topics covered

Demands for the determination of doping substances is rapidly increasing. Apparently no use of PLC is mentioned in the official testing methods of the International Olympics Committee For the enormous task of analysing some 2000 urine specimens for a broad variety of banned substances, GC is the primary technique although HPLC is used to screen for most diuretics, caffeine and stimulants that cannot be analysed by GC Samples that are 'positive' from the first quick test are then submitted to GC–MS for further assessment As most tests are qualitative, PLC would be helpful Combination of the advantages of HPLC and PLC can help to reduce the analysis time. Some doping agents, such as ephedrine, methylamphetanine, stimulants and sympathomimetic amines, were determined by OPLC on silica gel plates with butanol–chloroform–ethyl methyl ketone–glacial acetic acid–water (25 17 8 6 4) by Gulyas et al [362] Detection was carried out at 210 nm and the detection limit for caffeine was 1 μg Validation of the method is needed as many samples can be simultaneously determined (Fig 9)

For toxicological screening analysis TLC is a valuable technique because of its low cost and simplicity and the selectivity of the detection reagents Conventional and modern PLC are used for the separation and determination of cannabinoids [363,364]. A densitometric HPTLC method has been developed for the analysis of heroin and cocaine samples using silica gel plates [365]

The separation of heroin, monoacetylmorphine and morphine is needed to check the purity of illicit samples of heroin and in post-mortem blood analysis A rapid extraction procedure for codeine and morphine in whole blood has been

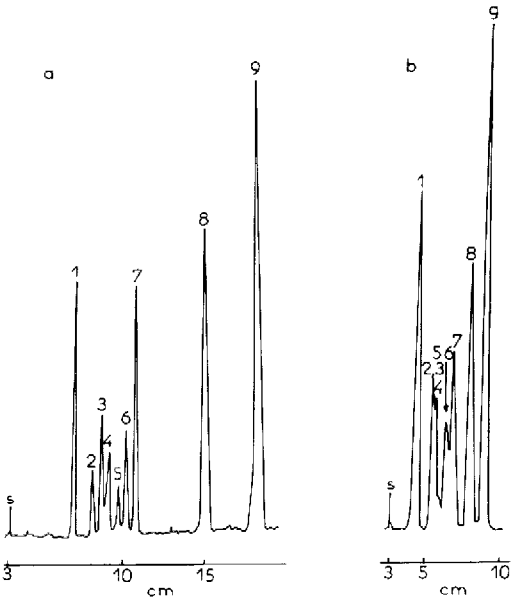


Fig 9 Separation of a mixture of doping agents Sorbent, HPTLC silica gel 60 F_{254}, eluent, *n*-butanol–chloroform–methyl ethyl ketone–water–acetic acid (25 17 8 4 6, v/v) (a) OPLC method separation distance, continuous development, development time, 25 min (b) Conventional TLC separation distance, 140 nm, development time, 95 min Peaks 1 = strychnine, 2 = ephedrine, 3 = metamphetamine, 4 = phenmetrazine, 5 = methylphenidate, 6 = amphetamine, 7 = desopimon, 8 = coramin, 9 = caffeine

proposed by Tebbett [366]. Red blood cells are fragmented by sonication and the blood sample is extracted by passing it through a Bond-Elut column TLC separation on silica gel with methylene chloride–isopropanol is performed very easily Thermal treatment in the presence of gaseous ammonia yields intense fluorescence of morphine and heroin, which thus can be easily detected in the presence of other drugs [367]

In forensic science various TLC methods have been applied Stead et al [368] published an important paper on standardized TLC systems for the identification of drugs and poisons. Dutt and Poh [369] gave the TLC profiles of some drugs A comparison of extraction methods for methylamphetamine and its metabolites in tissues was carried out by Inoue and Suzuki [370] In a report of two cases of suicide by amoxapine overdoses, Rohrig and Baker [371] advocated TLC for qualitative screening and GC for quantitation Owing to the structural similarity of cyclobenzaprine and amitryptiline, they could not be separated by either TLC, HPLC or GC [373]. As was emphasized by Bogusz et al [373], the type of biological matrix and the number of analysts may affect the inter-laboratory precision.

Hippuric acid is a metatobite of toluene and benzoic acid and also of ethylene

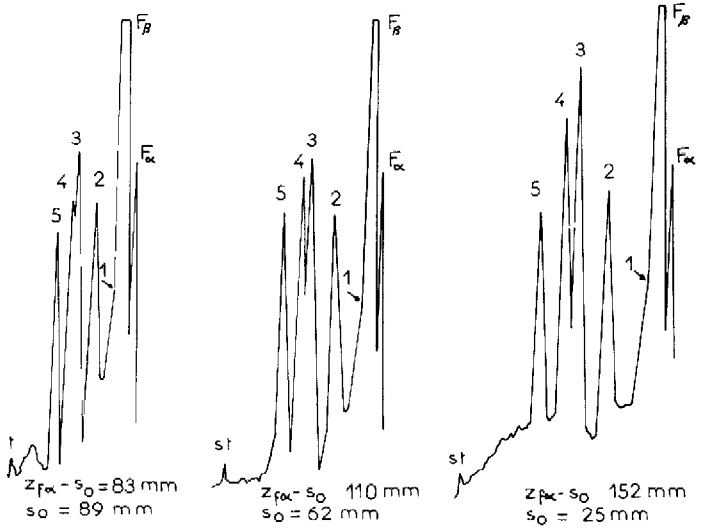


Fig 10 Separation of dimedone adducts of formaldehyde and other aliphatic aldehydes by RP-OPLC at different development distances Chromatographic conditions layer, RP-18 HPTLC silica gel (Merck) with impregnated edges, eluent, acetonitrile–0 005 *M* potassium dihydrogenphosphate (4 6, v/v), CHROMPRES 10 separation chamber (Labor-MIM, Budapest, Hungary), external pressure on the membrane 1 0 MPa, different separation distance, standards, 500 ng each, detection, 264 nm using Zeiss Opton PMQ III chromatogram spectrophotometer Peaks 1 = dimedone, 2 = acetaldemethone, 3 = formaldemethone, 4 = propionalmethone, 5 = butyraldemethone F_α and F_β = solvent fronts, S = starting point, s_o = starting distance, Z_f = α-front distance

glycol. The analysis of hippuric acid is a test of exposure Samples of urine are submitted to TLC on silica gel plates with chloroform–methanol (60 40) or on C_{18} plates with ethanol–water–acetic acid (65 35 1) Fluorescence quenching permits the detection of 0.5 μg [374]

According to recent investigations, formaldehyde is a mutagenic and carcinogenic substance Fig 10 shows the separation of formaldehyde and other alipathic aldehydes as the dimedone adducts using an RP chromatoplate (RP-OPLC) and different development lengths The method is suitable for the identification and determination of these compounds in samples of biological origin

5 CONCLUSION

GC and HPLC have gained wide acceptance as they are fully instrumentalized, may provide high efficiencies and are equipped with excellent detectors PLC cannot compete and is disqualified when it is used as a very cheap technique A cheap technique means raw qualitative data. Fortunately, new advances in instrumentation have been achieved and in either the conventional

mode (essentially TLC) or the forced-flow mode (OPLC) PLC is able to provide quick and reliable data

Many of these improvements are not yet in current practice in the biomedical domain For example, silica gel is still too often considered as a universal layer and bonded silica phases are not fully and adequately exploited One remarkable exception is enantiomeric separations on chiral plates, which are rapidly expanding. Combination of TLC and MS is now possible but reports on the topic are not very numerous

Owing to the very complex nature of the solutes to be chromatographed in biomedicine, pretreatment and sample clean-up require great care Direct spotting of the sample on the plate followed by successive developments with different mixtures of solvents acting as sample extraction and pretreatment is obviously one of the major advantages of PLC To our knowledge, it is the only chromatographic technique where cells can be applied directly to the stationary phase In the same way, immunostaining or fluorescence line narrowing is impossible to achieve in HPLC but fairly easy in PLC

From the literature it is evident that in some areas (e.g., catecholamine quantitation) TLC has been superseded by HPLC When both selectivity and efficiency are required, as in amino acid and carbohydrate analysis, PLC is declining Conversely, in lipid analysis the unique features exhibited by PLC make it a very valuable technique that can be used either as a full separation and quantitation technique or as a preparative step prior to GC analysis

This complementary mode is obviously the best choice in the study of a disease or a metabolic pathway For example, information regarding the fatty acid composition of sphingomyelin from amniotic fluid of normal and diabetic pregnancies is gained from TLC and subsequent GC. On the other hand, quantitation of one class of lipids (e g , bile salts in gall-bladder bile) or porphyrin profile is conveniently achieved by PLC with the same sensitivity and reliability as with HPLC However, in spite of the multiple capabilities, the detection procedure is still too long (with the exception of OPLC) It may be expected that this drawback will be overcome with the advent of reliable videodensitometry

6 SUMMARY

In planar chromatography (PLC), the solvent flows through a layer either by means of capillary forces [conventional thin-layer chromatography (TLC)] or by a forced-flow system (over-pressured layer chromatography) Phases and instrumentation currently available are briefly examined The main applications in biomedicine are reviewed Although silica gel TLC plates still predominate, interest in other phases is increasing Unique detection features such as immunostaining are emphasized Although gas chromatography and high-performance liquid chromatography have superseded TLC in the analysis

of carbohydrates, amino acids and indole derivatives, interest in PLC continues to be high in lipid analysis

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