Separation & Purification Reviews
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597294

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Online publication date: 09 August 2003

To cite this Article Bladek, Jan and Neffe, Slawomir(2003) 'Application of Thin-Layer Chromatography in Clinical Chemistry ', Separation & Purification Reviews, 32: 1, 63 — 122
To link to this Article: DOI: 10.1081/SPM-120025026
URL: http://dx.doi.org/10.1081/SPM-120025026

SEPARATION AND PURIFICATION REVIEWS
Vol. 32, No. 1, pp. 61–120, 2003

Application of Thin-Layer Chromatography in Clinical Chemistry[#]

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INTRODUCTION

All chromatographic methods are suitable for examination of biological material, but only thin-layer chromatography (TLC) enables the simple, fast, cheap, and effective separation of these complex mixtures. Therefore TLC is one of the best known and thoroughly tested methods of the analysis of substances, which is significant in medical diagnosis. There are many handbooks (e.g., Fried and Sherma, 1999; Touchstone, 1990), book chapters (e.g., Bladek and Zdrojewski, 2000; Jain, 1996) and review publications (e.g., Anderson and Van Lente, 1995; Issaq and Jaenchen, 1991) that comprehensively summarized TLC applications in clinical chemistry. The TLC method has been used both as an analytical and as a preparative technique to solve

[#]Reprinted from *Separation Techniques in Clinical Chemistry*, Aboul-Enein, H. A., Ed.; Marcel Dekker, Inc.: New York, 2003; 433–487.
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DOI: 10.1081/SPM-120025026 1542-2119 (Print); 1542-2127 (Online)

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problems in biochemistry, hematology, immunology, and even molecular biology. TLC is characterized by high selectivity, and it enables separation of the analyte from interfering substances. A special advantage of TLC is its versatility. It offers a great number of different sorbents in commercial form, the possibility of plate spraying with more or less specific visualizing reagents or coupling TLC with different specific detectors, as well as the ability to use a broad range (as regards polarity and selectivity of solvent) of mobile phases. Another property of TLC is that many samples can be throughput because of the possibility of performing simultaneous separation of many samples. In modern thin-layer chromatography, sample application, development, and recording of the chromatograms is realized by fast automated procedures.

SHORT CHARACTERISTIC OF THE METHOD

Thin-layer chromatography is a variant of liquid chromatography. A stationary phase (plate) is prepared from uniform porous materials by binding them to a support (alumina, glass, or plastic foil). When an analyzed liquid mixture is applied to the plate, the chromatogram is developed. A mobile phase (solvents or their mixtures) traverses then along the stationary phase, and analytes (solutes) move across the plate. As in other chromatographic methods, separation occurs as the consequence of different interactions of the components to be separated with both a stationary and a mobile phase. Separated substances are retained with relation to the mobile phase front, and a measure of retention is the retardation factor $R_F = z_n/z_f$, where z_n and z_f mean the distance of the n^{th} analyte and mobile phase front travels from the start line. Unfortunately, R_F (or $R_F \times 100 = hR_F$) values are affected by a number of factors and therefore a standard analyte should be run at the same conditions as the test sample whenever possible. If it is not possible, a number (usually four) of reference compounds, whose R_F values are accurately known, can be run and then the corrected value of R_F can be estimated (cR_F).

Modern high-performance thin-layer chromatography (HPTLC) began with the introduction of high-efficiency adsorbents. HPTLC layers are fairly similar to standard TLC plates; the difference is primarily that the average size of the particles is smaller and the size distribution is tighter. The increase of efficiency results in fewer bonds broadening and hence improves resolution and makes for greater sensitivity of detection. In addition, the length of the chromatographic bed required (4–6 cm) is markedly less than that encountered in conventional TLC (10–15cm). In general, standard HPTLC (the terms TLC and HPTLC are now used interchangeably) is sufficient for the separation of most clinical chemistry interest analytes.

The four processes taking the most important role in TLC separations of clinical interest are adsorption, partitioning, ion exchange, and affinity interactions. Adsorption takes place between the substructure of the analyte and the various adsorptive centers of the sorbents. Solutes with high adsorption capacity bind more strongly to the stationary phase, resulting in enhanced retention. Partitioning results in different solubilities of the solute molecules in the mobile phase. Solutes with higher preference for the mobile phase elute before the compounds with lower solubility. Ion exchange refers to the electrostatic forces occurring between the dissociable polar parts of the solute molecules and the ionic centers of the sorbent surface. In case of affinity TLC, separations are carried out by specific interactions of biologically related agents.

Properties of the Chromatographic Systems

The separation probability is greatly enhanced by the proper selection of a chromatographic system for particular analytes. This term refers to both stationary and mobile phases. If the stationary phase is of higher polarity than the mobile phase the chromatographic system is called the normal phase (NP) system; if the mobile phase is of higher polarity, the system is named the reversed phase (RP) one. Representative separations of clinical interest in both NP and RP systems are presented in Table 1.

Stationary Phases

TLC stationary phases (silica gel, alumina, cellulose, polyamides, and other sorbents) consist of powder solids. They are chemically defined inorganic or organic materials with porous structure and relatively high specific surface area. Often modified adsorbents are recently used. During chemical modification, varied functional groups have been covalently attached to the chromatographic bed (usually silica gel material), which eliminates stripping of these groups by the mobile phase. Physical modification of adsorbent depends on their impregnation by various additives not miscible with mobile phase (additives are adsorbed on the support). Mixing the additive in the eluent used as a mobile phase can also modify the chromatographic system (dynamic modification), but the use of modified adsorbents led to an improvement of resolution.

Among inorganic sorbents, silica gel is certainly the best TLC adsorbent. It is a polar adsorbent applied as a stationary phase and as a support to obtain physically and (especially) chemically modified sorbents. Nonmodified silica gel is widely used for routine analyses in the NP system

Table 1. Representative examples of separations in NP and RP systems.

No.	Type of analyte	Matrix	Chrom. system	Fundamental goal of analysis	Ref.
1	Biologically active amines	Urine	NP	Metabolism of the catecholamines and serotonin measurements	Dworzak and Hauk, (1971)
2	Bile acids	Bile	RP	Separation of unconjugated bile acids and their glycine- and taurine-amidated, 3-sulfated, 3-glucosylated and 3-glucuronidated conjugates	Momose et al. (1998)
		Feces	NP	Determinations of major unconjugated bile acids (cholic, chenodeoxycholic, deoxycholic, urodeoxycholic, and lithocholic acids) in human stool specimens	Kindel et al. (1989)
3	Carbohydrates	Urine	NP	The lactulose determination of patients with cystic fibrosis	Flick et al. (1987)
4	Vitamins	Blood	NP	A reliable procedure for the joint analysis of vitamin E (tocopherols), cholesterol, and phospholipids in the concurrent samples of human platelets in human cultured endothelial cells	Leray et al. (1997)

5	Lipids	Serum	RP	Determination of cholesterol sulfate and dehydroepiandrosterone sulfate	Serizawa et al, (1987)
		Brain	NP	Micropreparative isolation and purification of ganglosides	Müthing and Heitmann, (1993)
		Lung tissue	NP	Separations of lung phospholipids from matrix and their determinations	Krahn, (1987)
6	Enzymes	Urine	NP	Adenylosuccinase deficiency measurements (detection of succinyloaminoimidazolecarboxamide riboside and succinyloadenosine)	Wadman et al. (1986)
7	Porphyrins and their precursors	Urine, feces		Porphyria diagnosis. Evaluation of porphyrin	Henderson, (1989)
8	Alkaloids and drugs	Urine	RP	Determination of nicotine and its main metabolite cotinine for smoking and passively smoking pregnant women	Tyrpień et al. (2001)
		Blood	NP	Analysis of cyclosporine and its metabolites in peripheral blood (monitoring of transplant patients)	Roesel and Kahan, (1987)
		Urine	NP	Detection of opioids, cocaine, and amphetamine	Wolff et al. (1990)
9	Inorganic substances	Bones, milk	NP	Separation and detection of heavy metal ions	Baranowska et al. (1996)

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for many biological mixtures. Alumina has a similar property but is rarely used. All aluminas tend to adsorb molecules of water from the surrounding atmosphere and thereby become deactivated. In comparison with silica gel they have somewhat larger adsorption affinity for carbon–carbon double bounds and better selectivity toward aromatic hydrocarbons and their derivatives. Alumina is also rarely used as a support material. Other inorganic sorbents such as inert silicon dioxides (diatomaceous earth or silicon dioxide) have found only limited application in clinical chemistry.

Celluloses (native or microcrystalline) are organic sorbents. They have a low specific surface area and are applied mainly in partition chromatography, especially for the separation of relatively polar compounds. Celluloses have primarily been used as a support material for the separation of polar substances by normal-phase liquid–liquid partition. Layers have been impregnated with buffers, chelating agents, metal ions, and other compounds. Celluloses impregnated with polyethylene imine (PEI) and polyphosphate (poly-P) or chemically modified celluloses (chemical bonded aminoethyl [AE], carboxymethyl [CM], etc.) have been recently used as ion exchange material. Polyamides (synthetic resins, mainly caprolactam or polyundecanamide) are also numbered among the organic sorbents. Polyamides show a high affinity and selectivity to the analytes that can form hydrogen bonds.

Separations of analytes in reversed-phase systems were originally carried out on silica gel or diatomaceous earth layers impregnated with a solution of paraffin, squalane, silicone oil, etc. Recently, silica gel with covalently bounded organic ligands on the surface is frequently used. Methyl (RP-2), octyl (RP-8), octadecyl (RP-18), and phenyl silicas have often been used as the nonpolar chemically bonded adsorbents. Other chemically bonded adsorbents (diol-, cyano-, and amino-modified) have hydrophilic properties. Amino layers have a polarity lower than silica gel and higher than cyano and diol layers. In this way the gap of selectivity of the extremely hydrophilic nonmodified silica gel and the nonpolar RP phases was bridged by medium polar reversed phases. The mechanisms of retention on chemically bonded reversed phases are not clearly elaborated, but R_F values for a series of solutes separated on nonpolar phases are usually reversed in comparison with the sequence on silica gel, if water constitutes a large proportion of the mobile phase. Of course, a proper separation on reversed phase is also possible if one uses entirely organic mixtures as the mobile phase. The medium polar phases can be used in both the adsorption and the reversed phase separation mode, depending on the properties of the mobile phase.

Stationary phases showing different retention characteristics from those of traditional NP and RP systems are also used in clinical applications. Chiral beads (reversed phase silica modified with Cu^{2+} and a chiral agent, e.g., β-cyclodextrin) are used for enantiomeric separation. The retention of solutes on such beads mainly depends on their steric parameters. The

affinity thin-layer chromatography (ATLC) is also very significant. As in other types of affinity chromatography, the ATLC uses interactions of biologically related agents. Chiral chromatography based on binding agents of a biological origin (cyclodextrin or immobilized protein) can be considered as the affinity method.

Mobile Phases

Organic or inorganic solvents and even solutes of strong acids or bases, ion paring, ion-exchanger agents, etc. can be used in TLC as mobile phases. It also is worth underlining that the mobile phase is evaporated after the development of the chromatograms and it does not interfere with the solute spots during the visualization process. These two facts increase enormously the choice of eluent to be used in TLC, but organic solvents or their two-, three-, or more component mixtures are the best option. In the case of partitioning chromatography, properties of solvents are described by the Hildebrand (Hildebrand and Scott, 1964) parameter of selectivity. It is a measure of the sum of disperse dipole–dipole and hydrogen-bonded interactions. In the case of adsorption chromatography, properties of solvents are defined as the elution strength. Of course, the elution strength is higher when the mobility of the solute is higher. This parameter is calculated for a given stationary system. Lists of solvents ranked according to their elution strength are called elutropic series. The best known of them is Snyder series (Snyder, 1974), which is linked to silica gel. It consists of 81 solvents grouped in eight classes. In laboratory practice, the Hildebrand parameter of selectivity and the elution strength are used to select the chromatographic systems to be used.

A number of mixtures with various solvent percentages can be used in clinical applications, and their compositions depend on the nature of the separated analytes. For example, four mixtures, 1) ethyl acetate–methanol–30% ammonia (85:10:15), 2) cyclohexane–toluene–diethyl amine (65:25:10), 3) ethyl acetate–chloroform (1:1), and 4) acetone, have been proposed for the separation of drugs and their metabolites on silica gel (Romano et al. 1994). Lipids can be very well separated by a mobile phase of strong elution strength (first elution) and by a less polar mixture in a second run. Amino acids can be well separated on silica gel with very polar mixtures such as *n*-butanol–acetone–acetic acid–water (3.5:3.5:1:2) or pyridine–acetone–ammonia–water (80:60:10:35).

Sample Preparation and Application

There is a generally accepted view in laboratory practice that the TLC stage of sample preparation is not so important because TLC is also a cleanup technique. This is usually an incorrect view, especially in the case of

clinical analysis, where analytes are to be found in plasma, serum or urine, sometimes whole blood, faeces, saliva, cerebrospinal fluid, gastric fluid or body tissues. These matrices are very complex mixtures and therefore sample preparation prior to TLC analysis cannot be omitted. The process of separation from biological samples and purification of analytes is usually realized by protein precipitation, dialysis, hydrolysis, ultrafiltration, dilution, liquid–liquid, or solid-phase extraction. Originally, the most common extraction method was liquid–liquid extraction, but in recent years a solid-phase extraction has became an increasingly popular method for isolation of compounds from biological matrices. Lyophilization, saponification, microwave processing and supercritical fluid extraction are less common.

The first step of separation by TLC is the application of the laboratory sample onto the plate. A line is drawn with a pencil parallel to, and 0.5–2 cm from, the bottom of the plate. The samples are spotted onto this starting line either in spots or in bands (application of bands usually results in better separation). Diverse types of capillaries were originally used for spotting of samples, but recently calibrated syringes or automated spotting equipment is used to apply precise amounts of the sample on the start line, in either spots or bands. The purpose of the separation (analytical or preparative) and the detection limit of the analytes frequently determine the sample volume. When a large volume of sample must be applied, the use of plates with preconcentrated zones or the automated spray-on technique is indispensable. The preconcentrate plate consists of two zones of different adsorbents, merging into each other but with a sharply defined boundary. One of them (the spotting zone about 3 cm long) has comparatively poor adsorptive properties, while another forms a plate for the analytical separation. Any size of spot placed on the spotting zone run in the mobile phase will become a sharp band before it gets to the analytical part of the plate. The use of the automated spray-on technique is more complicated. The sample is transferred onto the plate by means of a nitrogen or other inert gas stream. This will keep the starting zones as small as possible. Many application systems are in the market, which makes possible applying 50–500 μL of sample. Both methods improve the sensitivity of the detection and the separation efficiency.

Development Techniques

For the favorable solute separation a set of parameters (composition and characteristic of the chromatographic system, the manner of mobile phase movement, etc.) has been determined. The elution, in which the separation conditions are not changed throughout the time required for the sample separation, is defined as isocratic. In the case of a gradient elution, the mobile phase composition or its pH (rarely temperature or composition

of the adsorbent) has changed during the separation. Successful separations of many complex biological mixtures by gradient elution have demonstrated the utility of this technique. Important also is the manner of mobile phase transport. This can be divided into two groups: the capillary flow (CF) and the forced flow (FF) movement methods. CF development of chromatograms is carried out with capillary forces. In case of FF development an external force is required to move the mobile phase throughout the sorbent. The most important of them are overpressure layer chromatography (OPLC) and rotator planar chromatography (RPC). OPLC is more rapid than CF TLC and offers various possibilities to improve separations. RPC is mainly used as a preparative technique.

Classical Techniques of Development

The one- or two-dimensional ascending or horizontal techniques are usually applied in TLC. An ascending developing chamber consists of a glass tank that has ground edges at the top to make an airtight seal with the glass lid. The development of chromatograms can be carried out in the presence (saturated chambers) or absence (nonsaturated chambers) of a gas phase in equilibrium with the mobile phase. When the mobile phase is added to the ascending chamber, the TLC plate is placed in a vertical position so that the starting line is above the level of the mobile phase. The mobile phase rises because of capillary forces. Sandwich chambers are also used in which the TLC plate is clamped between two glass plates separated by a gasket. A solvent is passed on one edge of the plate from the reservoir. The advantage of this system is that it uses less mobile phase and provides a more quickly saturated vapor system. Two-dimensional separation can be useful when particular separations are needed. The plate is run in the same direction with the first mobile phase, dried, turned 90°, and run again using a different mobile (rarely stationary) phase. In clinical analytical practice, two-dimensional developments are frequently used (Figure 1). However, in such separation only one sample can be spotted on each plate.

Multiple Developments

Multiple techniques of separation involve the repeated development of the chromatogram with one or more mobile phases. Mobile phases are removed between successive developments. These techniques can be divided into three groups: 1) unidimensional; the chromatogram is developed repeatedly for the same length with the same mobile phase, 2) incremental; the same mobile phase is used, but the first development length (step) is shorter and each subsequent development is incremented, and 3) gradient multiple development. In the case of gradient elution the mobile

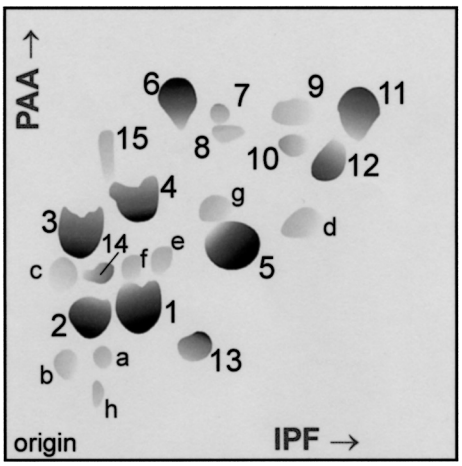

Figure 1. Two-dimensional chromatogram of amino acids in human urine. The sheets were developed by ascending migration with piridine–acetone–aqueous ammonium hydroxide–water (13:8.5:2.5:6, v/v; PAA) for 90 min, air-dried, turned through 90°, developed with isopropanol–formic acid–water (25:3:2, v/v; IPF). Spot notation: 1-glycine, 2-glutamine, 3-histidine, 4-serine, 5-alanine, 6-theronine, 7-tryptophan, 8-tyrosine, 9-phenylalanine, 10-methionine, 11-leucine/isoleucine, 12-valine, 13-glutamic acid, 14-lysine, 15-taurine; a-arginine, b-cystine/cysteine, c-methylhistidine, d-β-aminoisobutyric acid, e-homocitrulline, f-hydroxyprolin, g-proline, h-phosphoethanolamine. (From MC Hsieh, HK Berry. Detection of metabolic diseases by thin-layer chromatography. J Planar Chromatogr 2:118–123, 1992.)

phase composition changes for each (or for just a few) development step accompanying unidimensional or incremental development. Gradients of increasing solvent strength are preferable for complex samples spanning a wide polarity range. Decreasing solvent strength gradients are effective for simpler mixtures where a smaller separation capacity can be employed. Multiple development has some advantages over normal development. The most important of them are greater efficiency and separation capacity owing to the zone refocusing mechanism, optimum use of solvent selectivity, and improvement of sample detectability by scanning densitometry, owing to smaller dispersion spots or zones. Multiple chromatography is rarely required for the routine TLC separation in clinical chemistry because of isocratic separation that usually permits rapid analysis with highly reproducible R_F values. Unfortunately, many TLC screening methods must employ a multiple separation to deal with the wide range of hydrophobicity encountered among substances of interest to clinical chemistry.

Immunoassay Separation

The method combines the use of the solid-phase-bonded affinity ligand and conventional TLC and can be applied for both purification (preparative ATLC) and analysis (analytical ATLC) of the sample. Most ligands are of biological origin, but molecules of nonbiological origin (metal chelates, synthetic dyes) can also be used. There are many types of affinity chromatography. Bioaffinity chromatography includes any method that uses a biological molecule as the affinity ligand (nucleic acids, selectively retained DNA or RNA-binding proteins, and enzyme inhibitors and cofactors are used for enzyme binding). A special category is immunoaffinity TLC, in which the affinity ligand is an antibody or antibody-related agent (antibodies retained drugs, hormones, proteins, peptides, or viruses). In the laboratory practice the strip of a chromatographic plate in a discrete region, usually near the origin, is coated with affinity ligand. After injecting the sample on the start line, the chromatogram is developed by a mobile phase that has the proper pH value and does not wash covalently bonded affinity ligand. The solute that is complementary to the ligand is retained on the plate, while components not associated with the immunoreactive ligand migrate with the solvent front. Then the solvent that displaced solute from the strip or that promoted dissociation of the solute–ligand complex again develops the chromatogram. Sometimes the radiolabeled solute is analyzed and the latter step cannot be done.

Instrumentation of Developing Process

The term instrumental thin-layer chromatography is used to qualify the analytical process, in which steps occurs by appropriate apparatus. Instrumental TLC is becoming more frequently used in many clinical laboratories because the instrumentation improves the separation and makes determinations and the quality assurance necessary to maintain the validity of test results.

Automated multiple (usually gradient) development (AMD) is the most representative of CF instrumentation techniques. AMD equipment enables incremental gradient multiple development in which individual steps of separation (conditioning, development, and removal of mobile phase) is automated. The number of development steps, their length, and their solvent strength gradient are programmed before separation. A typical AMD separation is shown in Figure 2.

Progress in FF analytical separations has mainly been made as a result of overpressure instruments. In this technique the eluent is forced through the sorbent by means of a pump system using a desirable flow rate. Thanks

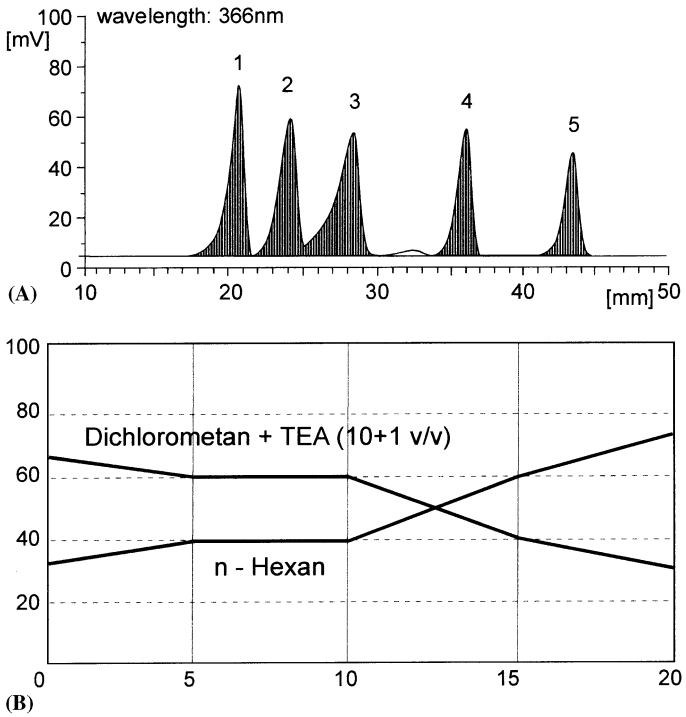

Figure 2. Separation of the biogenic amines (A) by use of gradient elution (B). Peaks from the left: 1-putrescine, 2-cadaverine, 3-histamine, 4-tyramine, 5-ephedrine. (From MH Vega, RF Saelzer, CE Figueroa, GG Ríos, VHM Jaramillo. Use of AMD HPTLC for analysis of biogenic amines in fish meal. J Planar Chromatogr 1:72–75, 1999.)

to these, OPLC offers decreases of the development time, insignificant band spreading, and improvement of detectability. OPLC can also be performed in two-dimensional mode. RPC is another TLC technique with FF elution employing the centrifugal force of a revolving rotor to move the mobile phase and separate components of mixtures.

Multimodal Separation Techniques

In order to improve separation and quantitative determination of analytes, the coupled chromatographic techniques (TLC–HPLC or TLC–GC) are used. The thin-layer and column liquid chromatography coupling can be performed in the TLC–HPLC or HPLC–TLC mode. In the former case, TLC is used as a cleanup technique (indirect coupling); spots

separated on TLC are scraped, and the analytes are dissolved in suitable solvents and then separated and quantified by HPLC. In the case of HPLC–TLC coupling (direct method), the suitable volume fractions of column eluent are mixed with nitrogen gas and sprayed as an aerosol onto the plate. TLC again separates analytes fractionated on column. Thanks to them, various separation mechanisms and specific visualization reactions can be used in one chromatographic process (Figure 3).

The TLC–GC coupling can be performed in direct mode, for example as the tabular TLC. The separation is carried out in a quartz tube, of which the inner surface is coated with a layer of inorganic adsorbent (usually silica gel). The tube is then driven through the scanning furnace and the separated fractions are consecutively vaporized, swept by carrier gas to the GC system. After the separation of analytes in the column, the GC detector analyses solutes. This method has been applied for analyses of phospholipids, glycolipids, bile acids, and other analytes of clinical interest. Indirect TLC–GC coupling is less complicated. As with the TLC–HPLC method, TLC is scaled down to a procedure for micropreparative separation of substances and serves as a cleanup technique.

Visualization and Quantitative Determination

The R_F value in comparison with those of the standard that had been developed under identical experimental conditions permits qualitative or semi-quantitative analysis. Colored substances, which have been separated on TLC plates, can be indirectly visualized. However, the majority of

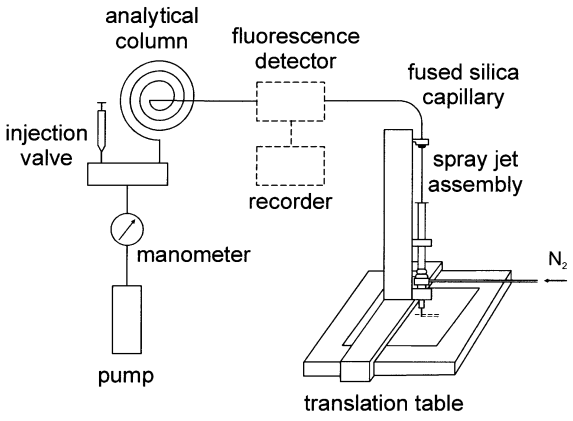

Figure 3. HPLC–TLC coupling. (From JW Hofstraat, S Griffioen, RJ van de Nesse, UATh Brinkman, C Gooijer, NH Velthorst. Coupling of narrow-bore column chromatography and thin layer chromatography. J Planar Chromatogr 3:220–226, 1988.)

solutes do not absorb light in the visible range and they must be detected by other methods. These methods usually have been divided into three groups: chemical, physical and physicochemical, and physiological–biological. Some of them can be used for quantitative determinations. Two basic techniques are available for the quantification of analytes. In the first of them (the densitometry in situ), the solutes are assayed directly on the layer. In the second one (the indirect method), analytes are removed from the layers and after the extraction assayed by other, sometimes very sophisticated, instrumental methods.

Chemical Methods of Visualization

In this method colorless compounds are converted into colored derivatives by use of the appropriate reagents. This can be performed before chromatographic development (the colored products are then separated) or after chromatography. Postchromatographic derivatization is the basic method of visualization especially when there is a specific reaction than can confirm the presence of particular analytes in the spot or band. The majority of chemical methods (both nonselective and selective) for derivatization of clinical chemistry interest were drawn up before 1990. Detailed information on this point can be found in the literature (Jork et al., 1990, 1994).

Physical and Physicochemical Methods of Visualization; UV Measurements

Among the physical methods of visualization, ultraviolet light (UV) measurements are used first of all. Substances containing UV chromophores can be qualitatively or quantitatively determined by UV absorption. Qualitative analysis occurs on the plates with a fluorescent (see below) indicator. The indicator placed on all surfaces of the plate emits visible light excited by UV (usually $\lambda = 254$ nm). Analyte absorbing UV prevents excitation (fluorescence of indicator is quenching) and become visible as a dark spot on the light background. Quantitative analyses can also perform on plates without fluorescent indicator by densitometer (densitometric evaluation enables rapid quantification of a lot of analytes). The separated substances are scanned with a flying spot of light or a fixed light beam in the form a rectangular slit. The absorbance of the light (UV) of the separated analytes is measured at appropriate wavelengths (maximum absorption) for the interest component. A diffusely reflected light is measured by the photosensor of the densitometer. The difference between the optical signal from the analyte-free background and that from an analyte

is usually correlated with the amount of the respective fraction of calibration standard separated on the TLC plate (Figure 4). It is obvious that a standard curve should be established on the same plate under the same conditions as for the analyte. Measurements of VIS absorption perform identically.

For the determination of very small amounts of substances, luminescence analyses have been adapted to TLC. In advance, phosphorescence analyses were used for measurements of some drugs, steroids, hydrocarbons, and other organic compounds separated on TLC plates. However, phosphorescence is rarely used in clinical chemistry, because cooling the analytical system or introducing so-called heavy atoms into the analyte is needed (an electronic oscillation in the molecule must be as small as possible). Recently, fluorescence measurements are most commonly used. They are more selective and sensitive than UV or VIS absorption measurements (the sample concentration can be 10^2-10^3 times lower). Nonfluorescing compounds do not usually interfere, so that analysis can be carried out also in the presence of such accompanying substances. Good results can also be obtained by conversion of nonfluorescing solutes into derivatives

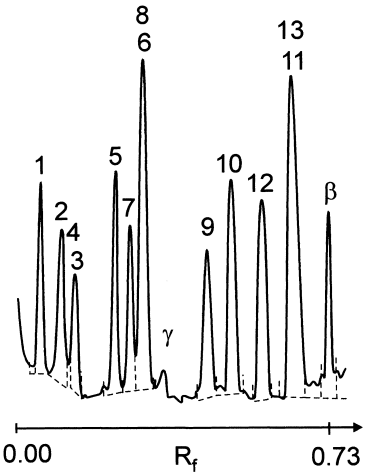

Figure 4. Chromatogram of benzodiazepines. Peak notation: 1-alprazolam, 2-midazolam, 3-chlordiazepoxide, 4-brotizolam, 5-carbamazepam, 6-cinolazepam, 7-nitrazepam, 8-clonazepam, 9-temazepam, 10-clobazam, 11-madazepam, 12-diazepam, 13-prazepam. (From E Hidvégi, S Perneczki, M Forstner. Data on the chromatographic behavior of solvent mixtures with similar solvent strength and selectivity. J Planar Chromatogr 6:414–419, 2000.)

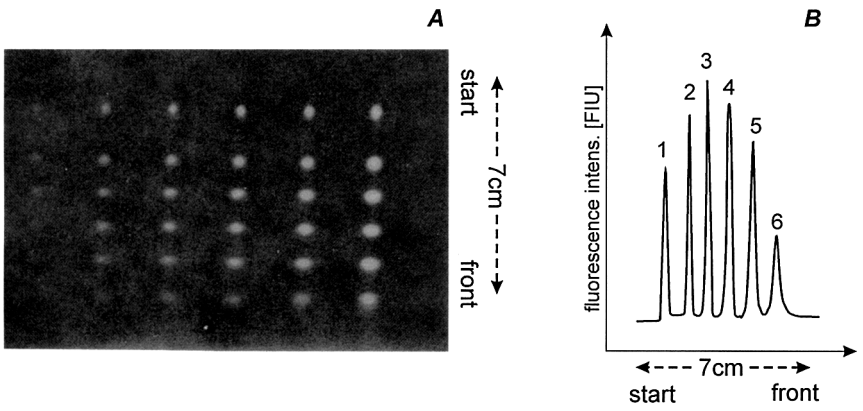

Figure 5. Fluorescence determinations: (A) Chromatogram of different concentrations of porphyrin standards, (B) densitogram. Peaks notation: 1-mesoporphyrin, 2-coproporphyrin, 3-pentaporphyrin, 4-hexaporphyrin, 5-heptaporphyrin, 6-uroporphyrin. (From A Junker-Buchheit, H Jork. Urinary porphyrins: ion-pair chromatography and fluorimetric determination. J Planar Chromatogr 3:214–219, 1988.)

showing fluorescence. Reagents that have proved helpful in this connection include dansyl chloride (amino acids, corticosteroids, and estrogens), dansyl hydrazine (reducing sugars), and fluorescamine (amino acids, amphetamine). Quantitative determinations of analytes depend on densitometric measurements of fluorescence intensity at appropriate excitation and emission wavelengths (Figure 5).

Isotope measurement is the method available for evaluation in the μg range, because the labeled substances generally make up only a few percent of the total isolated compound. The method is considered to be selective, since accompanying material displays no emission and does not interfere with the evaluation. The short-lived radioisotopes ^{35}S, ^{32}P, and ^{131}I are of special interest in clinical chemistry because they do not overburden the organism. Comparatively long-lived ^{36}Cl, ^{14}C, or ^{3}H are used to facilitate analysis. Bacteria or toxins can be directly labeled (see below) prior to performing assay or metabolically labeled. The three principal methods for measuring radioactivity of labeled solutes are: 1) autoradiography (an image is produced on x-ray or photographic film after exposing it to emissions from solutes), 2) in situ detection by radiation detectors, and 3) indirect zonal analysis (the plate is segmented or sectioned, adsorbent is removed from the plate, and the radioactivity is measured). All can be used for quantitative analysis. An example of radiochromatography applications is presented in Figure 6.

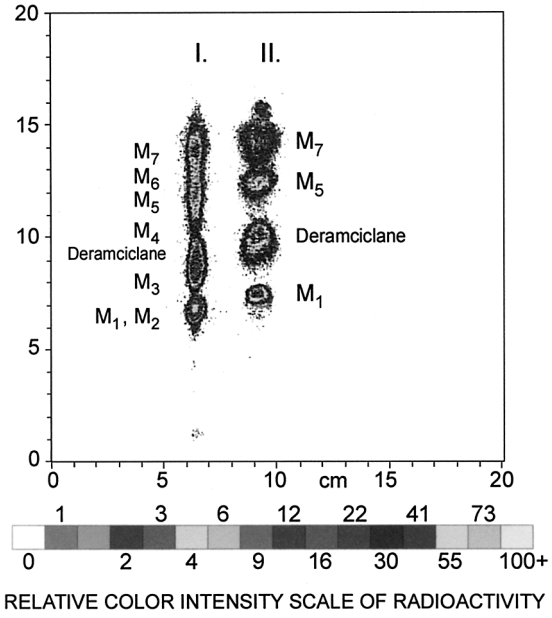

Figure 6. Digital autoradiography of two deramciclane metabolite samples obtained from nutrient matrix (I) and from liver cells (II). The different compounds found are indicated by M_1–M_7. (From K Ludányi, A Gömöry, I Klebovich, K Monostry, L Vareczkey, K Újszászy, K Vékey. Application of TLCFAB mass spectrometry in metabolism research. J Planar Chromatogr 2:90–96, 1997.)

Biological–Physiological Visualization

Biological–physiological determination depends, like affinity chromatography, on highly specific and sensitive interactions of biologically related agents. The three common versions of their method are bioautography, the overlay technique, and the direct enzymatic method.

In the case of bioautography, the test organisms are uniformly distributed in an agar or gelatin detector layer. As the solutes are separated on the plate, mobile phase residues are removed, layers are connected (sometimes through a blotting material), and the active solutes diffuse from the adsorbent into the detector layer. After some hours or even days of incubation, solutes display their inhibiting or beneficial action on the test organism. To have a result from these actions, radiolabeled substances are used or suitable reagent was either added to the detection layer or sprayed onto it after incubation (immunostaining TLC). The inhibition haloes or spots then appeared as light zones on a colored background or vice versa.

Overlay detection requires pretreatment of the chromatograms with plastic. The rationale for plastic coating is to prevent flaking of silica gel from the support. For the next step the plate is overlaid with a biological agent (antibody, toxin, etc.), incubated, washed with buffer and, if necessary, overlaid with the secondary detection agents (e.g., secondary labeled antibody). Then the plate is washed again and dried for autoradiography on x-ray film or treated for secondary color development. A parallel plate is usually developed in the same chamber under identical conditions and subjected to chemical detection.

Direct enzymatic reactions depend on spraying chromatograms with enzyme solutions. After incubation time, a suitable substrate is added and the reaction, as in the chemical detection, is followed visually.

All biological–physiological methods are highly specific, and inactive accompanying substances do not interfere in determinations. The methods are used to determine hormones, antibiotic and enzyme-inhibiting action of alkaloids, mycotoxins, pesticides, etc.

Multimodal Techniques of Detection

Identification of the analytes by the TLC method can be (as with other kinds of chromatography) ambiguous. One commonly thinks that substances with the same R_F value are identical. This assumption is not fully justified. The same retardation factor informs only about a huge probability of identity but it does mean absolute detection. The UV or VIS absorption spectra are not selective enough, and structurally similar substances cannot generally be distinguished. Moreover, the chromophore group is needed for detection based on UV or VIS absorption. More information can decidedly be obtained by coupling TLC with modern spectroscopic methods. Several methods have been developed for coupling TLC with spectrometric techniques and their applications for clinical chemistry. These include both indirect (off-line) and direct (on-line) methods. Indirect methods are probably the most readily implemented way to couple TLC with other spectroscopic methods. Standard nondedicated spectrometers can be used, and scraping off a TLC spot and dissolving the analyte in a suitable solvent do not require special equipment. Unfortunately, indirect methods are usually time-consuming and have a potential for loss of the sample.

These days the combination of TLC-Fourier transformation infrared (FTIR) and TLC-mass spectrometry (MS) are established in clinical chemistry. Raman spectroscopy signals are very weak and the sensitivity is too low for regular use. The quality of the FTIR or MS spectra is sufficient for discrimination between closely related substances. An advantage of these methods is detecting also nonabsorbing UV or VIS substances.

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Radiometry (the measurement of the radioactivity of isotopes) is also frequently used in clinical practice.

Direct coupling FTIR spectroscopy was introduced in 1989 by Glauninger et al. (1989). The principle of the method depends on scanning the plate fixed onto a computer controlled *x*,*y*-stage with an IR beam in a diffuse reflectance infrared Fourier transformation (DRIFT) unit. Of course, difficulties arise because of IR absorbance of the stationary phase. Commercially available plates (e.g., 50% silica gel and 50% magnesium tungstate) evaluate only the region between 3550 cm^{-1} and 1370 cm^{-1}, but this is enough to resolve many problems in clinical chemistry (Stahlmann, 1999). An example of an advantage resulting from direct TLC-FTIR coupling is presented in Figure 7.

TLCMS coupling does not require a special adsorbent, but the interface is needed for introduction of the analytes separated with TLC into the MS detector. The one- or two-dimensional systems spatially resolved analyses are used (thermal evaporation of the analytes to a gas stream connected to the mass spectrometer or a direct-insertion probe into fast atom bombardment [FAB] or secondary ion [SI] sources of mass spectrometer). In this method analytes are sputtered from adsorbent by impact of the high-

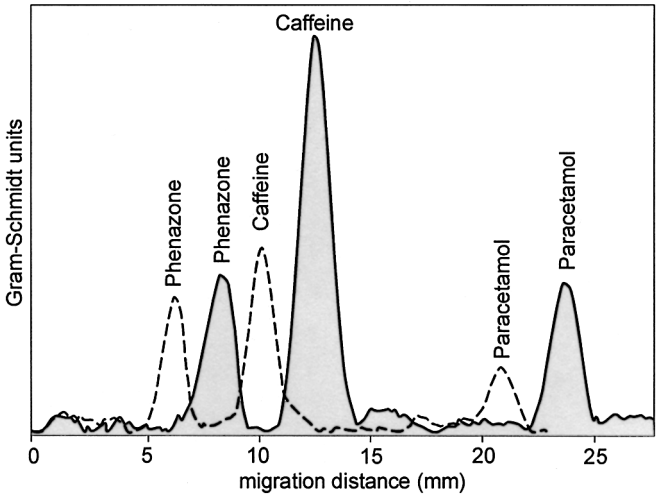

Figure 7. HPTLC-FTIR on-line coupling. Gram–Schmidt chromatogram of test substances on 1:1 mixed silica gel 60-magnesium tungstate (---) and on silica gel (—). (From GK Bauer, AM Pfeifer, HE Hauk, K-A Kovar. Development of an optimized sorbent for direct HPTLC-FTIR on-line coupling. J Planar Chromatogr 2:84–89, 1998.)

energy molecule stream. Liquid matrices such as glycerol or triethanolamine can be applied to the chromatogram before FAB or SI desorption. Recently matrix-assisted laser-desorption ionization (MALDI) was adapted to TLC. The method depends on pressing a previously prepared layer of matrix crystals into the TLC plate and elution of spots from the TLC plate to the MALDI layer via capillary action. A good example of MALDI

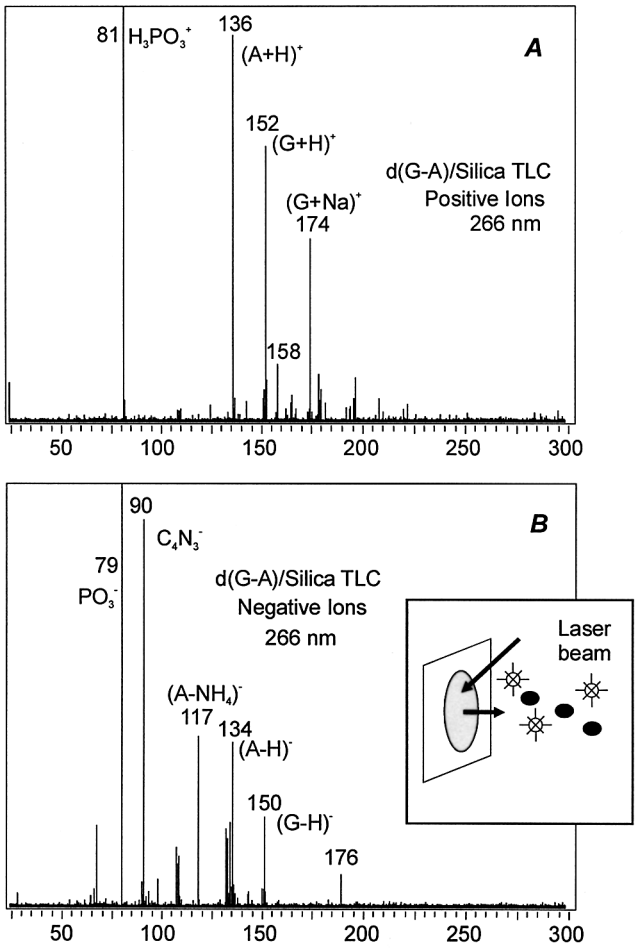

Figure 8. TLC-MS on-line coupling. Positive (A) and negative (B) ion laser desorption mass spectra of the dinucleotide d(G-A) obtained directly from a silica gel plate. (From KL Busch. Coupling thin-layer chromatography with mass spectrometry. J Planar Chromatogr 2:72–79, 1992.)

applications in clinical chemistry is the paper by Isbell and co-workers (1999). They proposed a methodology for the detection of pg quantities of nucleotides directly from TLC plates without the use of radioactive labeling. The matrix-assisted laser-desorption ionization–time of flight MS (MALDI-TOFMS) method is now proposed (Crecelius et al., 2000; Mechl and Hercules, 2000). It is a hybrid TLC-MALDI plate in which a silica layer and a MALDI layer are configured adjacently on a common backing material. Advantages of TLC-MS coupling are presented in Figure 8. TLC-MS-MS coupling provides further advantages by giving more detailed information for particular ions.

APPLICATIONS

TLC is widely used in clinical chemistry for the detection of many substances, such as amino acids, carbohydrates, drugs and their metabolites, lipids, and porphyrins or prostaglandin in various biological specimens, which are significant in medical diagnosis. Research papers connected with this problem can be divided into two fundamental groups. The first one concerns investigations of the method itself, usually done with standards. In these investigations, separation techniques and visualization methods are improved, and the principles of quantitative determinations are studied. The other group involves investigation of applications. As a result of these studies, a method describing the treatment of biological sample from its collection until complete information on the analytes or their metabolism products is obtained. In such investigations the extraction and cleanup technique, the chromatographic system, and the detection methods are developed, and the efficiency of each stage for the analytical process is optimized. Both of these applications are widely represented in the literature; applications presented in this part are limited to works that illustrate the capabilities of the method as well as its practicability.

Amino Acids

TLC has proved useful for screening and quantitative analysis of amino acids in urine and blood samples. The meaning of such analyses is crucial because early estimation of free amino acids in biological fluids and tissues may prevent neurological damage and mental retardation in young infants with inborn errors of amino acid metabolism (Edwards et al. 1988; Wu, 1991).

Almost all separations of untreated amino acids are performed in NP systems. Four stationary phases most commonly used for such separations are cellulose, silica gel, impregnated adsorbents, and ion exchangers. In the

case of silica gel, two- or three-component mixtures of solvents usually carry out separation. Acetone, methanol, water, acetic acid, and formic acids, sometimes with the addition of ammonia or pyridine, are most commonly used as the components of mobile phases. The basic amino acids showed smaller R_F values than did acidic ones, because of amino group and acidic group of silica gel interaction. Impregnated silica gel, silanized, or octadecyl modified silica gels are used for some separations. Different metal ions or dodecylbenzensulfonic acid are most commonly used as impregnating agents at various concentrations. Mobile phases are then similar to those used on silica gel. Cellulose ion exchangers and ion exchange resins used for amino acid separations need eluents with an adequate ionic strength. The most widely used reagent for qualitative and quantitative assessment of nontreated amino acids is ninhydrin reagent.

A widely employed method for the separation of enantiomeric pairs of amino acids (amino acids are optically active) is chiral separation. It may be performed with β- or γ- cyclodextrin attached to dimethylpolysiloxane or on conventional stationary phases by the inclusion of chiral additive into the mobile phase.

Determinations of *N*-terminal amino acids have a dominant meaning in clinical chemistry and are applied for peptide structure researches. In such analyses, prechromatographic derivatization is usually used. Dansyl and 3-phenyl-2-thiohydantoine derivatives are most commonly investigated. Separations are carried out on silica gel or polyamide layers.

Deyl (1986) presented applications of liquid chromatography for the profiling of amino acids in body fluids and tissues. The article includes many references of TLC technique. The review by Bhushan (1991) summarizes the application of TLC in the analysis of amino acids and their derivatives. Breakdown of some examples of TLC applications for amino acid research is presented in Table 2.

Listing the applications of TLC for amino acid analyses, it is impossible to pass over the works presented by Yahya and coworkers (1995; (Zakiah et al., 1995). They examined 404 urine samples to find whether inborn errors of amino acid metabolism could be the main cause of death among the children. Patients were aged between 11 days and 6 years. Of these, 41 percent had aminoacidurias. Very interesting are the works of Marklova (1997). They applied two-dimensional TLC for the diagnosis of inherited metabolic diseases. Analyses of amino acids, sugars, oligosaccharides, and organic acids were carried out on silica gel and cellulose with different solvents. HPTLC and HPLC were also used for the detection of defects in the metabolism of tryptophan (Marklova et al., 2000). HPTLC was suitable at the beginning of the investigation. An HPLC method with isocratic elution and spectrometric detection was used at the next

Table 2. Examples of TLC application for amino acid analyses.

Analyte	Matrix	Chromatographic system		Visualization and quantification	Ref.
		Stationary phase	Mobile phase (v/v)		
Chain and aromatic amino acids	Serum	Polyamide	Two-dimensional: I benzene-acetic acid (9:1.7), II formic acid-water (1.5:100)	UV_{254} detection, fluorometric determination after elution	Li et al. (1985)
Phosphotyrosine, phosphoserine, phosphothreonine	Protein hydrolyzates	Cellulose	Propionic acid-1M NH_3-isopropanol (45:17.5:17.5)	Ninhydrin reagent	Neuffeld et al. (1989)
Homocysteine	Blood	Cellulose	2-mercaptoethanol	Ninhydrin reagent	Ohtake et al. (1995)
3-phenyl-2-thiohydantoine derivatives of 16 amino acids	—	Silica gel	Three phases: I pyridine-benzene (5:40), II methanol-carbon tetrachloride (1:20), III acetone-dichloromethane (3:80)	Iodine vapors	Bushan et al. (1994)
Proline and hydroxyproline	Different biological samples	Impregnated silica gel	Isopropanol-water (7:3)	Ninhydrin reagent, autoradiography	De Maglio and Svanberg (1996)
20 main protein amino acids	—	Silica gel (single and double plates)	OPLC separation: butanol-acetonitrile-0.005 M potassium dihydrogen phosphate-acetic acid (1:5:3:1)	Ninhydrin reagent, quantification at 490 nm	Tyihak et al. (1998)

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step, when pathological findings were to be confirmed and the individual metabolites quantified. The first method enables the assessment of tryptophan, 5-hydroxyindolylacetic acid, indolylacetic acid, indolylacryloylglycine, and indolylacrylic acid and its possible precursors, namely, indolyllactic and indolylpropionic acids. The second procedure was intended for the monitoring of anthranilic, 3-hydroxyanthranilic, kynurenic and xanthurenic acids, kynurenine, 3-hydroxykynurenine and indoxyl-sulfate.

Drugs

The most important applications of TLC are the screening of hard intoxication or drug abuse and pharmacokinetic studies. The term hard intoxication is applied to the determination of harmful effects, arising in the short time after the introduction a large dose of poison to the body. Drug abuse refers to the administration of a drug or other biologically active substance in order to produce a pharmacological effect unrelated to medical therapy. Assays of poisons and abused drugs may be required in a variety of settings, but the most urgent requests are associated with clinical toxicology. This fact has necessitated the development of rapid and sensitive methods for the detection of opiates, barbiturates, benzodiazepines, amphetamines, cannabinols, and like substances in biological samples. The common use of TLC in such analyses is due to its ability to provide necessary data. Pharmacokinetic studies of drugs include drug concentration measurements and evaluation of the efficiency of new methods of therapy. The pharmacological action of many drugs depends not on the amount taken but on concentration in the blood. Relations between these two values (dose and therapeutic concentration) depend on the drug and often have an individual character. Therefore one of the tasks of clinical chemistry is to establish relationships between the concentration of the drug in blood and its dosage. Evaluation of the efficiency of new therapy methods mainly concerns drugs newly introduced into clinical medicine. The object of investigation is then not only the concentration of the drug in blood but also its assimilation and excretion, harmfulness, metabolism, etc. Wilson (1996) critically reviewed planar chromatography from the viewpoint of drug analysis in biological fluids. The capabilities of the various techniques of TLC and their advantages and disadvantages were discussed.

Screening of Hard Intoxication and Drug Abuse

The older diagnostic tests using TLC identification of analytes was mainly based on comparison of R_F values and on visualization with the specific color reactions. Tests for qualitative analyses of poisons and

different drugs have been known for many years. The Committee for Systematic Toxicological Analysis of the International Association of Forensic Toxicologists (TIAFT) approved a test for the separation of 1600 toxicologically relevant substances (International Association of Forensic Toxicology, 1987). The test is based on separating power measurements (database of reduced hR_F values is done) in ten standardized chromatographic systems (Table 3).

Chromogenic reagents are usually applied for the detection of drugs. Derivatization allows selective confirmation of amphetamine and primary amines (ninhydrin), barbiturates (diphenyl carbazone or mercuric sulphate), cocaine, antidepressants and antihistamines (iodoplatinate), narcotic analgesics (Dragendorff reagent), cannabinols (Fast blue B salt), etc. Especially useful for screening investigations are kits such as the Toxi·Prep (TP) kit, proposed by Steinberg and coworkers (1997a). The method is based on TLC and involves five major steps: solid phase extraction, concentration, spotting, development of chromatograms, and detection. The kits have been found to be particularly useful for the analysis of basic and neutral drugs. Some of the newest applications of TLC for drug screening are presented in Table 4.

Table 3. Systems for drug screening approved by the committee for systematic toxicological analysis of the international association of forensic toxicologists.

Analytes	Chromatographic system		Separation technique
	Stationary phase	Mobile phase (v/v)	
Acidic and neutral drugs	Silica gel	Chloroform–acetone (8:2) Ethylacetate Chloroform-methanol(9:1)	Unsaturated chamber
Acidic, basic, and neutral drugs	Silica gel	Ethyl acetate–methanol–conc. ammonia (85:10:5)	
Basic drugs	Silica gel	Methanol	Saturated chamber
		Methanol-*n*-butanol (6:4 + 0.1 mol/L NaBr)	
	Silica gel impregnated with potassium hydroxide	Methanol–conc. ammonia (100:1.5) Cyclohexane–toluene–diethylamine (75:15:10) Chloroform–methanol (9:1) Acetone	Unsaturated chamber

Table 4. Screening of hard intoxication and drug abuse in urine; examples of applications.

Analyte	Chromatographic system		Visualization and quantification	Ref.
	Stationary phase	Mobile phase (v/v)		
Opioids, cocaine, and amphetamine	Silica gel	Two mobile phases based on chloroform, methanol, and benzene	Iodoplatinate reagent	Wolff et al. (1990)
Salicilates	Silica gel	Benzene–acetic acid–diethylether–methanol (60:9:30:5)	Immunoassay	Kincaid et al. (1991)
Cocaethylene and cocaine	Silica gel	Hexane:toluene:diethylamine (65:20:5)	Iodoplatinate reagent	Bailey (1994)
Diazepam, nitrazepam, estazolam	Silica gel	Chloroform–benzene–acetone–methanol (5:5:3:1)	Densitometry at 300 or 200 nm	Simulataneous determination of diazepam, nitrazepam, estazolam in human urine by thin-layer chromatography (1996)

Metamphetamine	C-18	Ethyl acetate–ethanol–concentrated ammonia (36:2:2)	Fast Black K salt	Klimes and Pilarova (1996)
Amphetamine and its major metabolites	Silica gel	Toluene–acetone–20% ammonia–ethanol (45:45:7:3)	HPTLC-FTIR direct coupling	Pisternick et al. (1997)
Acidic and neutral drugs Basic, amfoteric, and quaternary drugs	C-18 Silica gel C-18	Methanol–water (13:35) Toluene–acetone–ethanol–conc. ammonia Methanol–water–conc. HCl	Fast Black K salt, Iodoplatinate, Dragendorff, Marquis, and Salkovsky reagents	Ojanperä et al. (1999)

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Performance of different techniques used to detect drug abuse in urine (based on external quality assessment) was presented in the literature. Twenty-five samples of lyophilized urine were analyzed by an average of 95 laboratories (Wilson et al., 1991). Samples contained mixtures of analytes and included replicated concentrations of morphine, methadone, amphetamine, and cocaine at 0, 1, 2, and 5 mg/L and of benzoylecgonine at 0, 0.4, 1, 2, and 4 mg/L. It turned out that some chromatographic techniques are inadequate for detecting morphine, amphetamine, cocaine, and benzoylecgonine at lower concentrations of the analytes studied. Gas–liquid chromatography was least sensitive for morphine; TLC was least sensitive for the other analytes. Few significant differences in specificity were detected between techniques, although significant interference from structurally related compounds was demonstrated in assays of morphine, methadone, and amphetamine.

Jain (2000) examined the utility of TLC for detection of opioids and benzodiazepines among drug addicts seeking treatment. Over a period of 5 performance years (1991–1995), 6055 urine samples were analyzed for opioids (morphine, codeine, buprenorphine, dextropropoxyphene, pentazocine) and benzodiazepines (diazepam, nitrazepam) by TLC. Out of all the drug tests ($n = 9922$) carried out, 24% of the drugs had been used during the past 72 h. Averaged across all drugs, the detection rates corresponding to 24, 48, and 72 h by TLC were 37%, 36%, and 31% respectively. A high percentage of negative TLC results were observed in these samples. Moderate sensitivity of the TLC assay procedure, low consumption of drug, short time between drug use and urine collection, and drug use history of the subjects obtained from multiple sources led to high negative results. These findings suggest that all the TLC negative results also need further confirmation by an alternative, more sensitive technique in a clinical setting.

Trying to make the drug abuse testing program more meaningful, Brzezińska et al. (1999) proposed the TLC-MS method for screening of biological samples for drugs and metabolites. Several TLC systems for many compounds of toxicological interest were described. Portions of standard drug solution were applied to silica gel plates. Chromatograms were developed with six mobile phases and detected with one or more of 10 reagents. R_F values were corrected with use of four standards. Analytes were also transferred to the MS direct inlet system and evaporated at 200°C. EI mass spectra were measured at 100 eV in full-scan mode. In this way corrected R_F values and the eight most intense MS peaks for 493 drugs and their metabolites were obtained. These data were kept as a library in personal computer-based search system. The merits of the method have been confirmed in biological sample investigations. Drugs were isolated

from matrices by liquid–liquid extraction at pH 3 or 9 or by SPE. Extracts were spotted on TLC plates and spots (after developing of chromatograms) were extracted with methanol-CH_2Cl_2 (1:1) and examined by MS.

Pharmacokinetic Studies

Drug concentration monitoring and evaluating the efficiency of new methods of therapy also require the use of relatively simple, cheap, and rapid analytical methods. From an analytical point of view, it is a more complicated process than drug screening, since quantitative analyses are needed. Investigations are mainly performed on blood, plasma, or serum samples. Example applications of TLC for such analyses are presented in Table 5.

Many investigators have confirmed the usefulness of TLC applications for pharmacokinetic studies. Forgacs and Cserhati (1995) studied the interaction of eight commercial anticancer drugs with human serum albumin by charge transfer RPTLC in neutral, acidic, and basic solutions. Calculations of the relative strength of interaction and the discussion of the effect of pH and the presence of mono- and divalent cations on the strength of anticancer drugs to interact with human serum albumin are presented. Le Roux and coworkers (1992) determined salbutamol concentrations by HPTLC chromatography in the sera of two sets of 10 volunteers at hourly intervals for 6 h after taking one 8 mg slow-release tablet. The influence of time lapse in processing of serum samples, i.e., centrifugation, extraction, and chromatography, was studied. A statistical significant instability of salbutamol in the sera of patients was found that was not present in standard drug-free serum samples spiked with salbutamol and used for construction of standard curves. Otsubo et al. (1995) developed a rapid and sensitive method of identifying benzodiazepines and zopiclone in human serum. The drugs were developed and separated on plates for 8 to 11 min and detected by means of UV radiation and color. Each drug was accurately identified by means of the values of hR_F and the spot color in three systems. The detection limit of the benzodiazepines in serum was 0.1–0.4 μg/mL, except for cloxazolam and haloxazolam. The sensitivity was increased about tenfold over the conventional method. Authors suggested that the HPTLC system is useful for the initial detection and identification of these drugs in emergencies. Lind et al. described the measurement of urinary ifosfamide, isophosphoramide, mustard, dechloroethyl ifosfamide, and carboxyifosfamide using HPTLC with photography (Lind et al., 1990). The technique was also used to demonstrate the large interindividual variation in the ifosfamide metabolic profile of patients receiving the drug

Table 5. Pharmacokinetic studies.

Analyte	Matrix	Chromatographic system		Quantification	Ref.
		Stationary phase	Mobile phase (v/v)		
Sumatriptan (sulphonoamide) and its metabolite	Blood, serum	Ten TLC systems recommended by TIAFT		UV_{254}, GC-MS	Rochholz et al. (1995)
Sinomenine	Serum	Silica gel	Chloroform-methanol (19:2)	Densitometry at 275 nm	Brzezińska et al. (1999)
Deramciclane (a new anxiolytic drug) and metabolites	Different body fluids	Silica gel	Butanol–acetic acid–water (4:1:1)	MS-MS (offline coupling)	Ludanyi et al. (1997)
Gentamycin	Plasma	Silica gel	Chloroform–methanol–20% ammonia (24:22:15)	NDB-Cl reagent, fluorodensitometry at 436 nm	Bhogte et al. (1997)
5-Methoxypsoralen	Serum	C-18	Methanol–water (4:1)	Fluorodensitometry at 490 nm	Mignot et al. (1997)
Psychosedative drugs	Serum	Silica gel	Heptane–chloroform–ethanol–ethyl acetate (10:4:4:3)	Densitometry (UV absorption)	Zhang (1998)
Theophylline	Plasma	Silica gel	Toluene–isopropanol–acetic acid (16:2:1)	Densitometry (UV absorption)	Jamshidi et al. (1999)
Tinidazole	Serum	Silica gel	Chloroform–acetonitrile–aceticacid (60:40:2)	Densitometry (UV at 320 nm)	Guermouche et al. (1999)

as single-agent therapy for non-small-cell lung cancer. In addition, oral administration was shown to result in higher levels of these metabolites in the urine. Fractionation of the ifosfamide dose over several days resulted in increasing levels of metabolites in the urine, consistent with autoinduction of ifosfamide metabolism.

The pharmacokinetics and metabolism of cyclophosphamide were studied in nine pediatric patients (Tasso et al., 1992). Cyclophosphamide and its major metabolites were determined in plasma and urine using HPTLC photographic densitometry. Plasma samples were obtained from eight subjects and urine was collected from six children during a 24 h period after drug administration. Cyclophosphamide was nearly, if not completely, cleared from plasma 24 h after its administration. The plasma half-life of drug ranged from 2.15 to 8.15 h, and between 5.4 and 86.1% of the total delivered dose was recovered as unchanged drug in the urine. The major metabolites identified in plasma and urine were phosphoramide mustard and carboxyphosphamide. The study suggests that there is interpatient variability in the pharmacokinetics and metabolism of cyclophosphamide in pediatric patients. Boddy and Idle proposed (Boddy and Idle, 1992) a method for the determination of the anticancer drug ifosfamide and its principal metabolites in urine, plasma, and cerebrospinal fluid. The urine and fluid samples were absorbed onto Amberlite XAD-2 eluting the compounds of interest with methanol. Plasma was deproteinated using cold acetonitrile and centrifuging to yield a clear supernatant liquid. The eluate and supernatant were analyzed by TLC with spot visualization. The plates were photographed for subsequent densitometric analysis. The intraassay coefficient of variation for each compound in both urine and plasma was less than 10%, and the lower limit of detection was 1 μg/mL. The method provides means for determining the full spectrum of metabolic products of ifosfamide in-patients and will allow detailed investigation of variability in metabolism and pharmacokinetics of this drug. A rapid and sensitive HPTLC assay for the measurement of nimesulide in human plasma has been evaluated by Pandya et al. (1997). Analysis was performed on plasma containing known amounts of the drug, on drug-free plasma, and on plasma containing an unknown quantity of the drug. Known amounts of extract and nimesulide (100 and 200 ng, as external standard) were spotted on silica gel plates by an autosampler. Quantification was achieved using a densitometer. The recovery of the method was $97.10 \pm 2.22\%$. The method was applied for the determination of plasma levels and pharmacokinetic parameters of nimesulide after oral administration of two formulations (100 mg) in healthy volunteers. The authors proved that the method is a sensitive, economical, rapid, and specific assay for nimesulide in human plasma and is suitable for pharmacokinetic studies after therapeutic doses.

Carbohydrates

Carbohydrates are naturally occurring substances that contain mainly carbon, oxygen, and hydrogen. Mono-, di-, tri-, oligo-, and polysaccharides, ketose, tri-, tetra-, pento-, and hexose as well as reducing and nonreducing sugars have great importance in the life sciences. Several diseases are accompanied by the increased elimination of sugars of various groups in the urine and feces.

TLC is widely used in biomedical research and clinical laboratories for separation of carbohydrates in biological samples. Silica gel and silica gel impregnated with various inorganic ions, cellulose, polyamide, and amino-modified silica gel are the most popular stationary phases, which permits us to obtain satisfactory separations. Water is usually a mobile phase component because of the high water solubility of sugars. Owing to them the biological impurities are left on the start line. A few dozen chromogenic visualizing reagents can be applied for the visualization of carbohydrates. Reagent-free visualization proposed by Klaus et al. (1989) is also very interesting. Sugars separated on amino-modified silica gel give fluorescence spots after heating at 120–150°C.

Prosek and co-workers (1991) presented a review on TLC sample preparation, chromatographic systems, detection, and quantitative evaluation of carbohydrates. A breakdown of some interesting examples of TLC applications for clinical researches is presented in Table 6.

Lipids

The lipids occurring free or bound as lipoproteins comprise complex mixtures of different classes of compounds. They play a vital role in virtually all aspects of biological life. Disturbance in the lipid metabolism of the organism leads to various disorders. Analyses of lipids have therefore a great diagnostic value (Table 7).

Three kinds of adsorbents are recently used for lipid separations, namely classical silica gel, silica gel impregnated (mainly with silver—Ag TLC), and RP-modified silica gel. In case of silica gel separations, lipids with free carboxyl, keto, and hydroxyl groups give lower R_F values than those that are only fatty acid residues when petroleum ether or hexane (main components) and acetone or diethyl ether (polar modifiers) are used as mobile phases. Silica gel impregnated with silver ion is used to separate the molecular species of a single lipid class. It offers an effective means of lipid mixture fractionation into distinct fractions differing in the number of double bounds. Petroleum ether, hexane, toluene, and chloroform are then most often used as the major components of mobile phases. RP systems

(usually silica gel with chemically bounded octadecyl and a polar solvent such as acetone, acetonitrile, or water) are rarely used and only for the separation of individual classes of lipids.

Various types of development (one- and two-dimensional and multiple development) are applied. Of course, the use of two-dimensional and multiple TLC is particularly valuable for the separation of complex lipid mixtures. The most popular mobile phases (Fried, 1991a) used for the separation of lipids on silica gel are presented in Table 8.

Visualization is frequently carried out by solutions of chromogenic substances applied as spraying reagents. Aniline blue, bromophenol blue, helasol green, and alkaline blue are used to detect cholesteryl esters. Molybdic oxide and phosphomolybdic acid turned out to be very good reagents for phospholipids. Fluorescamine, aluminum chloride, and ferric chloride can be used for analyses of fatty acids. Glycolipids are usually detected with orcinol, sulfuric acid, and α-naphthol, ganglosides with resorcinol. These reagents are suitable both for nonmodified and modified adsorbents. When radiolabeled substrates are analysed, non-destructive reagents should be used. The I_2 vapors or fluorescamine spraying reagent does not affect the lipid and can be easily removed during the isolation processes. The most sensitive nondestructive dye for lipids also is primuline.

Many papers and review articles on TLC lipid separation are presented in the literature (e.g., Fried, 1991b; Touchstone, 1995); some new and representative works on this point and on the usefulness of the proposed solutions are presented below.

Neutral Lipids

Modern pathophysiological researches in the human cell demand the specific analysis of neutral lipids such as cholesterol, cholosetryl esters, and triglycerides. Conventional enzymatic or calorimetric assays, while quite suitable for classical clinical chemistry, are of limited sensitivity and specificity, and the major classes of neutral lipids have to be determined separately. These results can be achieved through TLC; nanogram quantities of cholesterol, cholesteryl esters, fatty acids, and triglycerides can be detected.

Absolute specificity and high accuracy are required for the quantitation of cholesterol in small biological samples, particularly in a limited number of cells. Asmis and coworkers (1997) proved that both can be achieved through TLC and phosphomolybdic acid staining, while the shortcomings of traditional spot detection are overcome by laser densitometry. The major advantage of the proposed technique is the concurrent assay of ng quantities of cholesterol, cholesteryl esters, and triglycerides. The assay is at least

Table 6. Examples of TLC application for carbohydrates analyses.

Analyte	Matrix	Chromatographic system		Visualization and quantification	Ref.
		Stationary phase	Mobile phase (v/v)		
Monosaccharides	Plasma membrane	Polyamide	Ethyl acetate–acetic acid–ethanol (80:8:10)	Calorimetric, after elution with water	Tang et al. (1985)
Galactose metabolites	Blood	NH_2-silica	Ethyl acetate, acetic acid, methanol, and water mixtures in different percentages	Enzyme immunostaining	Bowling and Brown (1986)
Oligosaccharides	Gangliosides	Polyamide, NH_2-silica	Ethyl acetate, acetic acid, methanol, and water mixtures in different percentages	Enzyme immunostaining	Higashi et al. (198)7
Different sugars	Glycosides	Silica gel	Two-dimensional development: (I) toluene–ethyloacetone–methanol (5:5:4), (II) toluene–ethyloacetone–methanol–formic acid (5:5:4:0.1)	Aniline-*o*-phtalic acid reagent	Wang and Ma (1989)

Carbohydrates	Different biological materials	NH_2-silica	Different solvent systems	Fluorescence at 360 nm after heating at 150°C	Klaus et al. (1991)
Reducing sugars	Protein hydrolyzates	Silica gel	Multiple development: (I) butanol–pyridine–water (16:5:4), (II) ethyl acetate–methanol–acetic acid–water (4:1:1:1)	*o*-Toluidine reagent and heating at 110°C	Morcol and Velander (1991)
Neutral sugars	Cell walls	Silica gel impregnated with phosphate buffer	Acetonitrile–amyl alcohol–water (6:2:2)	*N*-(1-Naphtyl)-ethylene diamine reagent and heating at 100°C	Batisse et al. (1992)
Sugars and maltodextrins	Human milk, biological fluids	Cellulose	Multiple development: (I) butanol–ethanol–water, (II) pyridine–ethyl acetate–acetic acid–water (5:5:1:3)	Silver nitrate, sodium thiosulfate, or Elson Morgan reagent	Boscher-Reig et al. (1992)
Oligosaccharides	Human milk	Anion exchanger	Ethyl acetate–acetic acid–water (5:5:4)	FAB-MS	Rudloff et al. (1996)

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Table 7. The main goal and results of lipid researches by TLC.

Class of lipid	Technique of measurements	The main goal and results of researches	Ref.
Lipids in plasma	TLC separation and ozonization of analyte	The elucidation of a biochemical criterion of the degree of metabolic disorders in children with insulin-dependent diabetes mellitus. It was shown that the pattern of unsaturation distribution in plasma lipid fractions might serve as a new biochemical criterion for metabolic disorders and decompensation in insulin-dependent diabetes mellitus.	Poznyak et al. (1996)
Corneum lipid	AMD-HPTLC separation and GC determination	Investigation of the internal stratum corneum lipid in relation to depth by extraction following one, three, or five stripping. A decrease in unsaturated free fatty acids and in the unsaturated/saturated chain ratio with depth was observed. A decrease in the ratios of free fatty acids to cholesterol and free fatty acids to ceramides with depth was also observed. Results: a decrease in the ratios of free fatty acids to cholesterol and free fatty acids to ceramides with depth confirmed the diagnostic importance of this level of stratum corneum lipids in skin barrier properties.	Bonte et al. (1997)

Cholesterol sulphate	HPTLC separation and densitometric determination	Measurement of blood plasma and erythrocyte membrane samples of patients suffering from diabetes and Down's syndrome. It was postulated that differences in cholesterol sulphate levels might contribute to changes of erythrocyte properties in these pathological states.	Przybylska et al. (1995)
Salivary lipids	TLC separation and densitometric determination	Research of the role of salivary lipids in oral health. A positive correlation between the body mass index and the level of saliva cholesterol concentration was found. It was shown that, in healthy adults, saliva cholesterol concentration reflects serum concentration to some extent and can be used to select individuals with high serum cholesterol levels.	Karjalainen et al. (1997)
Phospholipid and sphingomyelin	Immuno-TLC separation and determinations	Determination of pulmonary surfactant phospholipid/sphingomyelin ratio in human amniotic fluids. The method was applied to determine the surfactant phospholipid/sphingomyelin ratio in 20 μL of the amniotic fluids obtained at delivery. The amniotic fluids from women who delivered a baby suffering from respiratory distress syndrome were easily discriminated from the normal amniotic fluids.	Iwamori et al. (1996)

(*continued*)

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Table 7. Continued.

Class of lipid	Technique of measurements	The main goal and results of researches	Ref.
Sulfatides	HPTLC separation and densitometric determination	Evaluation of sulfatides in the urine of patients with metachromatic leukodystrophy deficiency. The amount of sulfatides is expressed in relation to sphingomyelin, which copurifies with sulfatides and better reflects the level of membrane lipids in urine than commonly used parameters (creatinine, urine volume, etc.). The method is also useful as a complementary analysis for other lipidoses with high excretion of sphingolipids in urine (e.g., Fabry disease).	Berna et al. (1999)
Gangliosides	Two-dimensional HPTLC and GC	Determination of gangliosides profile of cancer patients. The profiles of cancer patients were compared to those of the control group, revealing a significant increase in total lipid-bound sialic acid and a specific increase in polysialogangliosides in the patients with breast cancer. An increase was noted in the ratio of gangliosides of the b-series biosynthetic pathway over those of the a-series in the cancer sera, as compared to the controls. No unusual gangliosides were found in the mixture from breast cancer patients.	Wiesner and Sweeley (1995)

Table 8. Mobile phases for separations of lipids on silica gel.

Class of lipid	Technique of separation	Solvent system (v/v)
Neutral	One-dimensional	Petroleum–ether–diethyl ester–acetic acid (80:20:1)
	Double separation in the same direction	(I) Isopropyl ether–acetic acid (94:6), (II) petroleum ether–diethyl ether–acetic acid (90:10:1)
	Two-dimensional	(I) hexane–diethyl ether (8:2), (II) hexane–diethyl ether–methanol (7:2:1)
Phospholipids	One-dimensional	Chloroform–methanol–water (65:25:4)
	Double separation in the same direction	(I) Chloroform–methanol–water (65:25:4) (II) hexane–diethyl ether (4:1)
	Two-dimensional	(I) Chloroform–methanol–water (65:25:4), (II) *n*–butanol–acetic acid–water (6:2:2)
Glycolipids	One-dimensional	Chloroform–methanol–water (65:25:4)
	Two-dimensional	(I) Chloroform–methanol–7N ammonium hydroxide (65:30:4), (II) chloroform–methanol–acetic acid–water (170:25:25:6)
Gangliosides	One-dimensional	Chloroform–methanol–2.5 M aqueous ammonia (60:40:9)
	Two-dimensional	(I) Chloroform–methanol–0.2% aqueous $CaCl_2$ (60:35:8), (II) *n*–propanol–water–28% aqueous ammonia (75:25:5)

tenfold more sensitive than common TLC techniques and at least fourfold more sensitive than common enzymatic methods. The proposed low-cost assay is highly reproducible and may be particularly suitable for routine lipid analysis of a 10% aliquot of relatively small tissue and cell samples.

Nonesterified fatty acids (''free'' fatty acids) are usually not present in the free form. They occur in blood predominantly in association with albumins to which they are bound by electrostatic forces rather than covalent bonds. They play a significant role in the physiological control of carbohydrate metabolism. Serum lipid fatty acid compositions have been determined in diabetes, coronary disease, and artery disease, as well as renal disease. Esterified fatty acids are components of complex lipids such as glycerides and phospholipids. Sattler and coworkers presented (Sattler

et al., 1996) a simple, accurate, and fast procedure for quantitative analysis of fatty acids in simple lipid subclasses from different biological specimens. TLC fractionated lipid extracts of isolated plasma lipoproteins (very low-, low-, and high-density lipoproteins) into lipid subclasses on silica gel plates. Bands comigrating with lipid standards were scraped off under argon and subjected to direct in situ transesterification with BF_3/MeOH in the presence of the TLC adsorbent. Fatty acid methyl esters were subsequently extracted and quantified by capillary gas chromatography. Miwa and coworkers described (Miwa et al., 1996) a study on the assay of fatty acid compositions of individual phospholipids from non-insulin-dependent diabetes mellitus patients. Eight major phospholipids were separated by a TLC method with a one-dimensional developing system without any pretreatment of the plate. The fatty acids incorporated into each phospholipid class were analyzed by an improved HPLC method with a simple elution system, advantageous with respect to resolution and analysis time. The fatty acid compositions of individual phospholipids in platelets were investigated following administration of ethyl cis-5,8,11,14,17-eicosapentaenoate for more than 13 weeks to patients with non-insulin-dependent diabetes mellitus. The cis-5,8,11,14,17-eicosapentaenoic acid compositions of all phospholipid classes were significantly increased with decreasing platelet aggregation rates after the administration. These results suggested that the method provides the complete separation of individual phospholipids in sufficient amounts to allow fatty acid analysis on the isolated phospholipid moieties.

Alvarez et al. (1995) described a test for the determination of dipalmitoyl phosphatidylcholine (as free dipalmitoylglycerol) in amniotic fluid. Aliquots of amniotic fluid were hydrolyzed with *Bacilus cereus* phospholipase C, and the resulting diglycerides were analyzed by $AgNO_3$-modified HPTLC reflectance densitometry. This system provided resolution of dipalmitoyl phosphatidylcholine and palmitoylpalmitoleoylglycerol from other 1,2-diglycerides and cholesterol. The turnaround analysis time for triplicate aliquots of amniotic fluid was 40 min. Recoveries ranged between 90 and 98%. The author suggested that the method provides a quantitative, specific, highly reproducible, and fast turnaround means of dipalmitoyl phosphatidylcholine analysis in amniotic fluid.

Multistep TLC-MS has been developed for the quantification of neutral lipids and several phospholipids extracted from mammal cells and sera (White et al., 1998). The lipid classes were separated by a TLC procedure in different solvent systems. Resolved lipid bands were visualized by the lipophilic dye primulin and scanned by an automated laser-fluorescence detector. The mass of each band was determined by comparison band intensities of unknown samples with dilution curves of standards. The

majority of biological lipids could be resolved and quantified with these methods. Since the detection method is nondestructive, purified lipids could then be recovered by scraping the visualized bands and extracting the lipids from the silica. MS extracted lipids were also hydrolyzed to release acyl chains and acyl chain species and then determined by GC, which confirmed the structural identities of the recovered lipids. In contrast to classical two-dimensional TLC (which allows a good resolution of some lipid species but cannot be used to analyze more than a single experimental point per plate), multistep TLC allows the direct comparative analysis of multiple samples on a single TLC plate. The method provides a good resolution for the quantification of most classes of lipid species.

Complex Lipids

The researches on complex lipids have seen considerable increase in recent years. Glycolipids have been reported to be associated with differentiation, development, and organogenesis. These glycolipids have an active role in the formation of cataracts. The glycolipid biosynthesis pathway is initiated by the glucosyltransferase-catalyzed synthesis of glucosylceramide. The *n*-butyldeoxynojisimycin is an inhibitor of this synthesis and has been shown to be an inhibitor of HIV replication in vitro. After the pioneering work of Gluck et al. (1972 and Gluck and Kulovich, 1973) on the lecithin/sphingomyelin (*L*/*S*) ratio (the method was used for prediction of respiratory distress syndrome), TLC has become the most widely used technique for complex lipid analysis. The method has become the standard tool for resolution of ganglioside mixtures for analytical and preparative applications. The review by Müthing (1996) summarizes the application of TLC in the analysis of gangliosides up to 1995. Basic general techniques and special advice are given for successful separation of glycosphingolipids. New approaches concerning continuous and multiple development, and several preparative TLC methods, are also included. Emphasis is placed on TLC immunostaining and related techniques, i.e., practical applications of carbohydrate-specific antibodies, toxins and bacteria, viruses, lectins, and eukaryotic cells.

A new and simple method for purifying phospholipids and sphingolipids by using ''TLC blotting'' was established by Taki and coworkers (1994). Glycosphingolipids separated by two-dimensional thin-layer chromatography (TLC) were made visible with a primuline reagent, and then bands were marked with a colored pencil. TLC blotting to a polyvinylidene difluoride membrane, together with the color marks, then transferred the glycosphingolipids that separated onto the HPTLC plate. The marked areas were scraped, after which their glycosphingolipids were extracted

and monitored by TLC. By this method, 20 glycosphingolipids showing homogeneous bands on a HPTLC plate were isolated from the neutral glycosphingolipid fraction of human meconium. Moreover, 10 kinds of acidic glycosphingolipids were purified as homogeneous bands from the bovine acidic glycosphingolipid fraction. The yields of glycosphingolipids (13 different ones) ranged from 68 to 92%, the mean value was 82.3%. The authors suggested that the same procedure could also be used to purify phospholipids.

Müthing and Cacic (1997) described the comparative TLC immunostaining investigation of neutral glycosphingolipids (GSLs) and gangliosides from human skeletal and heart muscle. A panel of specific polyclonal and monoclonal antibodies as well as the GM1-specific choleragenoid was used for the overlay assays, combined with preceding neuraminidase treatment of gangliosides on TLC plates. This approach proved homologies but also quantitative and qualitative differences in the expression of ganglio, globo, and neolacto series neutral GSLs and gangliosides in these two types of striated muscle tissue within the same species. The main neutral GSL in skeletal muscle was LacCer, followed by GbOse3Cer, GbOse4Cer, nLcOse4Cer, and monohexosylceramide, whereas in heart muscle GbOse3-Cer and GbOse4Cer were the predominant neutral GSLs beside small quantities of LacCer, nLcOse4Cer, and monohexosylceramide. No ganglio series neutral GSLs and no Forssman GSL were found in either muscle tissue. GM3(Neu5Ac) was the major ganglioside, comprising almost 70% in skeletal and about 50% in cardiac muscle total gangliosides. GM2 was found in skeletal muscle only, while GD3 and GM1b-type gangliosides (GM1b and GD1 alpha) were undetectable in both tissues. GM1a-core gangliosides (GM1, GD1a, GD1b, and GT1b) showed somewhat quantitative differences in each muscle; lactosamine-containing IV3Neu5Ac-nLcOse4Cer was detected in both specimens. Neutral GSLs were identified in TLC runs corresponding to e.g. 0.1 g muscle wet weight (GbOse3Cer, GbOse4Cer), and gangliosides GM3 and GM2 were elucidated in runs which corresponded to 0.2 g muscle tissue. Only 0.02 g and 0.004 g wet weight aliquots were necessary for unequivocal identification of neolacto type and GM1 core gangliosides, respectively. Muscle is known for the lowest GSL concentration from all vertebrate tissues studied up to now. Using the overlay technique, reliable GSL composition could be revealed, even from small muscle probes on a suborcinol and subresorcinol detection level.

Analyses of phospholipids and their fatty acid composition from human intestinal mucosa were performed by a method elaborated to analyze the limited amount of sample with two-dimensional TLC followed by lipid–phosphorus determination (Nakanishi et al., 1994). Using this method, plasmenylethanolamine was detected in human intestinal mucosa and

accounted for about 7% of phospholipid in small and large intestinal mucosa. The amounts of polyunsaturated fatty acids of phosphatidylethanolamine were higher than those of other phosphoglycerides in intestinal mucosa; hence inflammation-related eicosanoids may originate from ethanolamine containing phospholipid.

Guittard et al. reported (Guittard et al., 1999) results for analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) of native glycosphingolipids (GSLs) after development on thin layer chromatographic plates and after heat transfer of the GSLs from the plates to several types of polymer membranes. The spectral quality is better for membrane-bound analytes, in terms of sensitivity, mass resolution, and background interference. The sensitivity gain compared with liquid secondary ion mass spectrometry (LSIMS) of GSLs on thin-layer plates is 1 or 2 orders of magnitude (detection limits of 5–50 pmol vs. 1–10 nmol). Resolution and mass accuracy (0.1%) are limited by the irregular membrane surfaces, and this effect cannot be entirely compensated by delayed extraction. The best results were obtained with a polyvinylidene difluoride (PVDF) P membrane, with irradiation from a nitrogen laser. Although the Nafion membrane could not be used for molecular weight profiling, its acidic character led to sample hydrolysis at the glycosidic linkages, thus yielding a series of fragments that could be used to determine the sequence of carbohydrate residues. Structural information could also be obtained by postsource decay (PSD) experiments on mass-selected precursor ions. Samples containing both neutral and acidic components were characterized in a 1:1 combination of 2,5-dihydroxybenzoic acid and 2-amino-5-nitropyridine. GSLs that exhibited binding to antibodies in an overlay assay on the TLC plate were transferred to membranes and analyzed by MALDI-TOFMS without interference from the antibody or the salts and buffers used during the binding and visualization steps. Taking advantage of the insights into sample preparation gained from these studies, future research will extend this approach to analysis by matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICRMS) with an external ion source.

Liu et al. compared (Liu et al., 1998) two analytical methods (TLC and IR spectroscopy) for prediction of respiratory distress syndrome (RDS) from amniotic fluid analysis. Samples of amniotic fluid were obtained by amniocentesis from 86 patients between the 28th and 41st week of pregnancy. A small volume, 35 μL, of amniotic fluid was used to acquire infrared spectra with a commercial spectrometer. The *L/S* ratio was determined separately by TLC. A calibration model was established using a partial least square regression analysis, which quantitatively correlated 145 IR spectra with the TLC-based *L/S* ratios; the correlation coefficient was

0.95. The model was then validated using a total of 111 spectra, which also showed a high correlation coefficient ($r = 0.91$). Based upon the clinical data associated with these samples, the prediction accuracy for the presence of RDS was 67% for TLC and 83.3% for IR. The accuracy for predicting the absence of RDS was 93.4% for TLC and 96.8% for IR. The authors concluded that IR spectroscopy may become the clinical method of choice for predicting RDS from amniotic fluid analysis.

The chromatographic behavior of molecular species of sphingomyelin on HPTLC was investigated by Ramstedt et al. (1999). Sphingomyelin gave a double band pattern on HPTLC plates developed using chloroform/methanol/acetic acid/water (25:15:4:2, v/v) or chloroform/methanol/water (25:10:1.1, v/v). HPTLC analysis of acyl chain–defined sphingomyelins showed that the R_F values increased linearly with the length of the *N*-linked acyl chain. A double-banded pattern was therefore seen for natural sphingomyelins with a bimodal fatty acid composition. Racemic sphingomyelins also gave a double band pattern on HPTLC, where the lower band represented the D-erythro and the upper bands the L-threo isomer. It was showed that D-erythro-*N*-16:0-dihydrosphingomyelin migrated faster on HPTLC than D-erythro-*N*-16:0-sphingomyelin. The upper and lower band sphingomyelins from two different cell lines (human skin fibroblasts and baby hamster kidney cells) were separately scraped off the HPTLC plates, and the fatty acid and long-chain base profiles were studied using GC-MS. The lower bands contained short-chain fatty acids and most of the fatty acids in the upper bands were long. The predominant long-chain base was sphingosine, which was found in both upper and lower bands, but sphinganine was found only in the upper bands. To conclude, there are at least three possible reasons for the sphingomyelin double bands on HPTLC; acyl chain length, long-chain base composition, and stereochemistry. These reasons might sometimes overlap, and therefore HPTLC alone is insufficient for complete analysis of the molecular species of sphingomyelin.

A method of simultaneous determination of ceramides and 1,2-diacylglycerol in tissues was developed using the latroscan, which combines TLC-FID techniques (Okumura et al., 1998). Because of the relatively low amounts of these components in tissues, the fraction of nonpolar lipids, which includes ceramides and glycerides, was eluted with chloroform/acetone mixture (3:1, v/v) through a silica acid column to eliminate the polar phospholipids. Development was carried out using three solvent systems in a four-step development technique. The relationship of the peak area ratio to the weight ratio compared with cholesteryl acetate added as an internal standard was linear. The amount of ceramides increased with incubation of rat heart homogenate and human erythrocyte membranes in the presence of

sphingomyelinase (E.C. 3.1.4.12). The latroscan TLC-FID system provided a quick and reliable assessment of ceramides and 1,2-diacylglycerol.

Enhancement in separation of gangliosides on HPTLC plates has been obtained by automated multiple development chromatography (Müthing and Ziehr, 1996). A less polar mixture of the standard solvent chloroform–methanol–20 mmol aqueous $CaCl_2$ (120:85:20, v/v) was used. Lowering the water content achieved separation of two complex monosialoganglioside fractions, isolated from murine YAC 1 T lymphoma and MDAY-D2 lymphoreticular cells. Threefold chromatography in the solvent chloroform–methanol–20 mmol aqueous $CaCl_2$ (120:85:14, v/v) resulted in TLC separation of GM1b-type gangliosides, substituted with C24 and C16 fatty acids and with Neu5Ac and Neu5Gc as well, which could not be achieved by unidirectional standard chromatography. In comparison with conventional single chromatography, the technique described allows high-resolution separation of extremely heterogeneous ganglioside mixtures and offers a convenient tool for both analytical and preparative TLC.

An efficiency assessment of a ganglioside assay procedure was carried out on human serum gangliosides from healthy subjects of different sexes and ages (Negroni et al., 1996). The analysis of the gangliosides extracted with chloroform/methanol and purified by lipid partitioning, ion exchange column chromatographic separation, and desalting procedures was performed by HPTLC followed by densitometric quantification. The yield of the procedure, expressed as radioactivity recovery, was determined by adding GM3 ganglioside, tritium labeled at the sialic acid acetyl group and at the C3 position of sphingosine, to the lyophilized serum or by associating it with the serum lipoproteins. Although the extraction and purification procedures were performed exactly as described, we found the radioactivity recovery to be variable (25–50%) and much lower than that proposed. Much of the radioactivity was found in the organic phase after lipid partitioning, while all the ganglioside purification steps led to some further loss. The recovery improved, after the introduction of some modifications to the procedure, reaching, 67–79%. The analyses on 33 samples of 5 mL showed a human serum ganglioside content of about 10 nmol/mL (as corrected for the recovery) and confirmed that GM3 ganglioside is the main component of the total serum ganglioside mixture.

A human strain of influenza virus (A, H1N1) was shown to bind in an unexpected way to leukocyte and other gangliosides in comparison with avian virus (A, H4N6) as assayed on TLC plates (Miller-Podraza et al., 2000). The human strain bound only to species with about 10 or more sugars, while the avian strain bound to a wide range of gangliosides including the 5-sugar gangliosides. By use of specific lectins, antibodies, and

FAB and MALDI-TOF MS, an attempt was made at preliminary identification of the sequences of leukocyte gangliosides recognized by the human strain. The virus binding pattern did not follow binding by VIM-2 monoclonal antibody and was not identical with binding by anti-sialyl Lewis x antibody. There was no binding by the virus of linear NeuAcalpha3- or NeuAcalpha6-containing gangliosides with up to seven monosaccharides per mol of ceramide. Active species were minor NeuAcalpha6-containing molecules with probably repeated HexHexNAc units and fucose branches. This investigation demonstrated marked distinctions in the recognition of gangliosides between avian and human influenza viruses. The data emphasize the importance of structural factors associated with more distant parts of the binding epitope and the complexity of carbohydrate recognition by human influenza viruses.

Other Applications

The analysis of porphyrins is important for diagnosis of the porphyries, diseases due to enzyme deficiencies in the heme biosynthetic way (porphyrins are formed as intermediates in the synthesis of heme). TLC is the most widely used technique for the routine analysis of porphyrins. Porphyrins were extracted from urine or feces and separated on silica gel with methanol, chloroform, methylene chloride, carbon tetrachloride, ethyl acetate, benzene, and toluene mixtures. The distinct porphyrin bands were observed by viewing the plate under longwave fluorescent light. Luo and Lim (1995) have studied by HPTLC, HPLC, and LSI MS the porphyrin metabolisms in human porphyria cutanea tarda (PCT) and in rats treated with hexachlorobenzene (HCB). The analyses of porphyrin metabolites in the urine, feces, and liver biopsies of patients with PCT have shown that apart from uroporphyrin I and III and their expected decarboxylation intermediates and products, a complex mixture of many other porphyrins are present. The new porphyrins (meso-hydroxyuroporphyrin III, beta-hydroxypropionic acid uroporphyrin III, hydroxyacetic acid uroporphyrin III, peroxyacetic acid uroporphyrin III, beta-hydroxyproionic acid heptacarboxylic acid porphyrin III, hydroxyacetic acid hepatocarboxylic porphyrin III, and peroxyacetic acid pentacarboxylic porphyrin III) were identified.

Estimation of neopterin in urine has become part of the examination in phenylketonuria, malignities, and immunodeficiency including AIDS and HIV infection. Tomsova and Juzova (1989) described a simple chromatographic method for estimation of neopterin, which does not call for HPLC or imported kits. The di-and tetra-hydroforms of neopterin are oxidized with MnO_2 and stable neopterin is obtained. The specimen is purified on a Dowex 50WX4 column and on C18 columns. Then thin-layer chromatography

(silica gel 60 F_{254}) was used in a mobile phase of ethyl acetate/isopropanol/1 n NH_4OH (35:45:25, v/v). Fluorescence was assessed on a densitometer; it was linear within the 5–200 ng range. The method has also been sufficiently sensitive for the estimation of normal neopterin excretion. The authors submit the results of estimations in controls, immunopathies, and AIDS.

Maddocks and MacLachlan (1991) used TLC for homocysteine detection. A new fluorescent thiol reagent, dansyl-aminophenylmercuric acetate (DAPMA), was applied to the diagnosis of homocystinuria. A disorder of homocysteine can be associated with vascular disease at an early age. DAPMA was added to urine containing metabisulphite, and the resulting fluorescent derivatives were extracted on a cyclohexyl silica column and separated by thin-layer chromatography. One hundred two coded samples were tested. The derivative of homocysteine was easily identified in samples from four children with homocystinuria but was absent from all samples from normal subjects and patients with unrelated disorders. Other thiols (cysteine, acetylcysteine, mercaptolactate, thiosulphate, and thiocyanate) were also identified in urine from healthy fasting subjects.

Steinberg et al. (1992) analyzed DNA adducts by TLC (adducts occur through environmental, therapeutic, dietary, oxygen stress, and ageing processes). The authors proved that a TLC technique can assess base composition and adduct formation. This requires labeling DNA by ''shotgun'' 5′-phosphorylation of representative ^{32}P-α-deoxyribonucleotide monophosphates. Subsequent 3′-monophosphate digest ''sister exchanges'' a radioactive $^{32}PO_4^{(2-)}$ to the neighboring cold nucleotide. Separation in two-dimensional polyethyleneimine-cellulose TLC is carried out in acetic acid, $(NH_4)_2SO_4$, and $(NH_4)HSO_4$. The technique was applied to control DNA, cold substitution of dUMP, methylation, depurination, and pBR322. This technique quantifies low molecular mass adducts and DNA integrity both in vivo and in vitro.

Reddy (1993) reviewed the ^{32}P-postlabeling methodology for analysis of DNA adducts derived from carcinogens containing one aromatic ring (e.g., safrole, styrene oxide, benzene metabolites, 1-nitrosoindole-3-acetonitrile) or a bulky nonaromatic moiety (e.g., mitomycin C, diaziquone). Six steps are involved: digestion of DNA to 3′-nucleotides, enrichment of adducts, 32P-labeling of adducts, separation of labeled adducts by TLC, detection, and quantitation. The first step, DNA digestion with micrococcal nuclease and spleen phosphodiesterase, is applicable to DNA modified with most carcinogens independent of their size and structure. Of the two commonly used procedures for enrichment of aromatic adducts in DNA digests, the nuclease P1 treatment is substantially more effective than butanol extraction for small aromatic and bulky nonaromatic adducts. For initial purification of

these adducts from unadducted material after ^{32}P-labeling, multidirectional polyethyleneimine (PEI)–cellulose TLC using 1 M sodium phosphate, pH 6.0, as the D1 solvent is not suitable, because they are not retained on PEI-cellulose under these conditions. A higher concentration of sodium phosphate (e.g., 2.3 M) or development with D1 and D3 solvents in the same direction helps to retain adducts of safrole and of benzene metabolites. Also, transfer of adducts from multiple cutouts above the origin after D1 chromatography, as adopted for analysis of I-compounds, is potentially applicable. However, initial purification by reverse-phase TLC, followed by in situ transfer and resolution by PEI-cellulose TLC, has been found to be most effective for these adducts. Reverse-phase TLC at 4°C or in a stronger salt solution further improves the retention of some adducts (e.g., mitomycin C and diaziquone adducts). For adduct separation by PEI-cellulose TLC, salt solutions with or without urea are used.

The halopyrimidine 5-bromo-2′-deoxyuridine (BUDR) can serve as one of many indicators of tumor malignity, complementary to histologic grade. Steinberg et al. developed a TLC technique (Steinberg et al., 1997b) that can assess tumor DNA base composition and analog BUDR incorporation, which vies with immunochemistry for BUDR. This requires postlabeling DNA by nick-translation and radioactive 5′-phosphorylation of representative ^{32}P-α-dNMPs (deoxynucleotide monophosphates). Subsequent 3′-monophosphate digest exchanges a radioactive $^{32}PO_4$ for the neighboring cold nucleotide. Separation in two-dimensional PEI-cellulose TLC is carried out in acetic acid, $(NH_4)_2SO_4$, and $(NH_4)HSO_4$. TLC of dNMPs was applied to control HeLa DNA, and HeLa cells receiving BUDR. BUDR is detected in 10(6) HeLa cells after 12–72 h incubation. Findings in HeLa DNA demonstrate normal TLC retention factors for all ^{32}P-dNMPs. This technique quantifies BUDR-which parallels the tumor S phase and serves as an indicator of the labeling index.

A rapid immunochromatographic method for qualitative and quantitative analysis of protein antigens was described (Birnbaum et al., 1992). The method is based on the ''sandwich'' assay format using monoclonal antibodies (Mabs) of two distinct specificities. Mabs of one specificity are covalently immobilized to a defined detection zone on a porous membrane, while Mabs of the other specificity are covalently coupled to blue latex particles, which serve as a label. The sample is mixed with the Mab-coated particles and allowed to react. The mixture is then passed along a porous membrane by capillary action past the Mabs in the detection zone. The zone binds the particles, which have antigen, bound to their surface, giving a blue color. Analysis is complete in less than 10 min, requires a minimum amount of sample (4 μL), and has a detection limit below the nanomolar range for the antigen we studied, human chorionic gonadotropin. Identifi-

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cation of protein phosphorylation sites is essential in order to evaluate the contribution of individual sites to the regulation of a particular protein by phosphorylation. Van der Geer and Hunter developed (van der Geer and Hunter, 1994) a method for the identification of phosphorylation sites. The method is based on the digestion of ^{32}P-labeled proteins with site-specific proteases and separation of the digestion products in two dimensions on thin-layer cellulose plates using electrophoresis in the first dimension followed by chromatography. This method is very sensitive, requiring only a few hundred ^{32}P-disintegrations per minute to obtain reproducible phosphopeptide maps. Laitinen et al. showed (Laitinen et al., 1996) the possibility of the application of TLC to the analysis of protein–ligand affinity.

A HCHO level in cells of animal and human tissues as well as in body fluids depends on the physiological state of an organism. Rożylstroko and Siembida, (1998) Różyło et al. (1998); decided to find out if there are changes of HCHO level in different physiological and pathological hard tissues of teeth. Obtained results showed that there was some regularity in the level of HCHO as far as similar physiological or pathological states are concerned. This was best seen in comparison with the obtained results with mean HCHO level of the studied groups of teeth. The increase of formaldehyde level in some rare pathological cases of teeth was observed.

A TLC method for the separation of heavy metals and their complexes with dithizone, 4-(2-pyridylazo) resorcinol, and ethylenediaminetetraacetic acid was devised, and conditions for the solid-phase extraction of heavy metal ions in human bones, in placenta and milk after microwave mineralization, and in air after alkaline absorption were elaborated (Baranowska et al., 1996). In the corrosively aggressive medium of the oral cavity, the use of identical dental alloys requires identification of the existing metal construction. One of the methods allowing this identification is TLC with anodic sampling (Zivko-Babic et al., 1998). Using a 4.5 V battery and suitable electrolytes, seven dental alloys for fixed and removable dentures based on cobalt were analyzed. Chromatograms of alloy samples were developed with a mixture of acetone and 2 M HCl. Scanning of the TLC spots produced chromatographic curves, and the area under the curve was proportional to the content of cobalt in the alloy studied. Regression analysis showed a very high coefficient of correlation ($r = 0.999$) between the area of the spot and the proportion of cobalt.

The interaction of 28 commercial pesticides with human and bovine serum albumin as well as with egg albumin was studied by charge-transfer reversed-phase thin-layer chromatography, and the relative strength of the interaction was calculated (Cserhati and Forgacs, 1995). It turned out that only one pesticide interacted with egg albumin, whereas the majority of

pesticides bound to both bovine and human serum albumins. Stepwise regression analysis proved that the hydrophobicity parameters of pesticides exert a significant impact on their capacity to bind to serum albumins. These findings support the hypothesis that the binding of pesticides to albumins may involve hydrophilic forces occurring between the corresponding apolar substructures of pesticides and amino acid side chains. No linear correlation was found between the capacities of human and bovine serum albumins to bind pesticides. TLC and twelve color reactions were proposed for identification of foreign pesticides (Muzhanovski et al., 1998). Solubility of the agents was studied, optimal common extracting selected, and a universal method for isolation and identification of 19 new pesticides tried on the liver. The sensitivity and specificity of the method was assessed.

Analysis of mycolic acids by TLC (Leite et al., 1998) is employed by several laboratories worldwide as a method for fast identification of mycobacteria. This method was introduced in Brazil in 1992 as a routine identification technique. The method allowed earlier differentiation of *M. avium* complex-MAC (mycolic acids I, IV, and VI) from *M. simiae* (acids I, II, and IV), both with similar biochemical properties. The method also permitted to distinguish *M. fortuitum* (acids I and V) from *M. chelonae* (acids I and II) and to detect mixed mycobacterial infection cases as *M. tuberculosis* with MAC and *M. fortuitum* with MAC. Experience shows that mycolic acid TLC is an easy, reliable, fast, and inexpensive method, an important tool to put together conventional mycobacteria identification methods.

A TLC method has been developed for confirming results from the determination of aflatoxin M_1 in human urine (Skarkova and Ostry, 2000). Urine samples were cleaned on immunoaffinity columns and analyzed by means of immunochemical methods. A result higher than 5 ng/L urine was confirmed by instrumental HPTLC on silica gel layers with fluorescence detection. Chloroform/acetone/2-propanol (85:10:5, v/v) was used as a mobile phase. The chromatogram was scanned in reflectance mode at $\lambda = 366$ nm with a $\lambda = 400$ nm measuring filter. Twofold enhancement of the sensitivity of the HPTLC method was achieved by immersion of the chromatographic plate in a solution of paraffin oil in hexane. Recoveries were 75–85% in the range 20–100 ng/L urine. The limit of quantification in urine was 5 ng/L. Validation of the method was performed according to the principles used for HPTLC methods.

An autoradiographic technique for quantification of ^{10}B containing compounds used for neutron capture therapy is described (Schremmer and Noonan, 1987). Instead of applying solutions of $Cs_2B_{12}H_{11}SH$ and its oxidation products directly to solid-state nuclear track detectors, diethylaminoethyl cellulose TLC plates were utilized as sample matrices. The

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plates are juxtaposed with Lexan polycarbonate detectors and irradiated in a beam of thermal neutrons. The detectors are then chemically etched, and the resultant tracks counted with an optoelectronic image analyzer. Sensitivity to boron-10 in solution reaches the 1 pg/μL level, or 1 ppb. In heparinized blood samples, 100 pg $^{10}B/\mu L$ was detected. This TLC matrix method has the advantage that sample plates can be reanalyzed under different reactor conditions to optimize detector response to the boron-10 carrier material. Track etch/TLC allows quantification of the purity of boron neutron capture therapy compounds by utilizing the above method with TLC plates developed in solvent systems that resolve $Cs_2B_{12}H_{11}SH$ and its oxidative analogs. Detectors irradiated in juxtaposition to the thin-layer chromatograms are chemically etched, and the tracks are counted in the sample lane from the origin of the plate to the solvent front. A graphic depiction of the number of tracks per field yields a quantitative analysis of compound purity.

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