DEVELOPMENT OF ELECTROSPUN NANOFIBERS FOR THIN LAYER CHROMATOGRAPHY

By

Kosit Su-utha

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

Program of Pharmaceutical Sciences

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การพัฒนาเส้นใยนาโนโดยวิธีอิเล็กโตรสปินนิ่งสำหรับโครมาโทกราฟีแบบชั้นบาง

โดย

นายโฆษิต สุอุตะ

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The Graduate School, Silpakorn University has approved and accredited the thesis title of “Development of Electrospun Nanofibers for Thin Layer Chromatography” submitted by MR. Kosit Su-utha as a partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

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Thin layer chromatography is a simple, quick and inexpensive procedure for the qualitative and quantitative analysis. Typically, its separation media is prepared by spreading slurry of stationary phase such as silica gel on an unreactive carrier sheet made of glass, aluminum foil or plastic to form an adsorbent layer. In this study, a novel fabrication technique called electrospinning method was investigated and used for the preparation of the thin layer chromatographic plates from cellulose acetate(CA). The influence of parameters related to the polymers solution and electrospinning process on the properties of electrospun mat was studied. Subsequently, the electrospun fiber plates were used to separate steroid mixture consisting of dexamethasone and prednisolone using methanol-water as mobile phase. It was found that under the electric field of 17.5 kV/15 cm, uniform fibers were obtained from 17 % (w/v) cellulose acetate solution in 2:1 (v/v) acetone-Dimethylacetamide solvent, whereas cellulose acetate at the same concentration which was dissolved in 1:1 and 1:2 (v/v) acetone-Dimethylacetamide produced fibers with beads. Both fiber and bead diameter increased with the increasing spinning rate of spinning. The thickness of fiber mats also increased with the increasing spinning time and flow rate of spinning process. When the CA on aluminium sheets was used for the chromatography, it was found that electrospun plates could be successfully applied for the steroid separation with the highest resolution when 40:60 (v/v) water–methanol was used as a mobile phase. However, the migration behavior and retention time was influenced by the morphology and thickness of fibers. In most cases, the developing time was shorter for beaded fibers than smooth fibers. In the study the electrospun plates were also used for the resolution of chiral propranolol drugs. However, the separation was not successful and required further development.

Program of Pharmaceutical Science Graduate School, Silpakorn University Academic Year 2010

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51361204 : สาขาวิชาวิทยาการทางเภสัชศาสตร์

คำสำคัญ : โครมาโทกราฟีแบบชั้นบาง / อิเล็กโตรสปัน / สเตียรอยด์

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โครมาโทกราฟีแบบชั้นบางเป็นกระบวนการที่สะดวก, รวดเร็ว และ ประหยัด สำหรับการวิเคราะห์ในเชิงคุณภาพและปริมาณ โดยทั่วไปตัวกลางที่ใช้ในการแยกสารถูกเตรียมจากการเกลี่ยวัฏภาคคงที่ เช่น ซิลิก้า บนแผ่นรองซึ่งเฉื่อยต่อปฏิกิริยาของ แก้ว, อะลูมิเนียม และ พลาสติก จะได้ชั้นตัวดูดซับบนแผ่นรองขึ้น ในการศึกษานี้เทคนิคใหม่ในการเตรียมเส้นใยที่เรียกว่า กระบวนการอิเล็กโตรสปินนิ่งถูกนำมาศึกษา และใช้สำหรับการเตรียมแผ่นโครมาโทกราฟีแบบชั้นบางจากเซลลูโลสอะซิเตท(CA) ปัจจัยที่สัมพันธ์กับสารละลายโพลิเมอร์ และกระบวนการอิเล็กโตรสปินนิ่งต่อคุณสมบัติของแผ่นอิเล็กโตรสปัน ได้ถูกทำการศึกษา ซึ่งทำให้ได้แผ่นเส้นใยอิเล็กโตรสปันที่ใช้สำหรับแยกสารสเตียรอยด์ผสมของเด็กซาเมทาโซน และเพรดนิโซโลน โดยใช้เมทานอลและน้ำเป็นวัฏภาคเคลื่อนที่ พบว่าภายใต้สนามไฟฟ้าที่ 17 กิโลโวลต์/15 เซนติเมตร จะได้เส้นใยที่สม่ำเสมอจาก สารละลายเซลลูโลสอะซิเตท 17 % (w/v) ในตัวทำละลายผสมของอะซิโตนและไดเมทิลอะเซตาไมด์ในสัดส่วน 2:1 (v/v) ในขณะที่สารละลายเซลลูโลสอะซิเตท ที่ความเข้มข้นเท่ากันนี้ในตัวทำละลายผสมของอะซิโตนและไดเมทิวอะเซตาไมด์ในสัดส่วน 1:1 และ 1:2 ให้เส้นใยที่มีเม็ดบีสต์ ทั้งเส้นใยและเม็ดบีสต์มีขนาดเส้นผ่านศูนย์กลางเพิ่มขึ้นโดยการเพิ่มอัตราการพ่นของกระบวนการสปินนิ่ง ส่วนความหนาของแผ่นเส้นใยเพิ่มขึ้นได้ทั้งจากการเพิ่มอัตราการพ่น และระยะเวลาในการสปินนิ่งด้วยเช่นกัน เมื่อนำแผ่นCA บนอะลูมิเนียมมาใช้เพื่อเป็นโครมาโทกราฟีแบบชั้นบางแล้วพบว่าสามารถแยกสารสเตียรอยด์ผสมด้วยประสิทธิภาพการแยกสูงสุดที่วัฏภาคเคลื่อนที่เป็นน้ำ-เมทานอล ในอัตราส่วน 40:60 (v/v) อย่างไรก็ตามลักษณะของการเคลื่อนที่ และระยะเวลาที่ใช้ในการแยกสารสเตียรอยด์ผสมนั้นยังขึ้นอยู่กับลักษณะรูปร่างและความหนาของเส้นใยอีกด้วย ซึ่งโดยส่วนใหญ่ระยะเวลาที่ในการแยกสารสเตียรอยด์ผสมของแผ่นเส้นใยที่มีเม็ดบีสต์จะน้อยกว่าแผ่นเส้นใยสม่ำเสมอ การศึกษานี้ยังได้ทำการศึกษาการแยกไครัลผสมของยาโพรพาโนรอล แต่ไม่สามารถทำการแยกได้และยังคงต้องอาศัยการพัฒนาต่อไปอีก

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ลายมือชื่อนักศึกษา..

ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ 1. .... 2. ....

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CHAPTER 1
INTRODUCTION

1. Statement and significance of the research problem

Pharmaceutical industry is growing day by day with the aim to develop new drugs extracted from natural products or produced from synthetically chemical substances, but one thing always remains constant, that is, the product should be as pure as possible to guarantee the drug quality and safety. As a result, the analysis and assessment of drug purity is mandatory in the pharmaceutical process.

Among various analytical techniques, thin layer chromatography (TLC) is still one of the most popular mean for the analysis of pharmaceuticals and drugs with the evidence supported by the published literatures from 2008 to 2011 (Wagner et al. 2008 : 587; Hubicka et al. 2009 : 408; Latha et al. 2011 : 40). In those reports, numerous examples are provided for qualitative and quantitative TLC analysis of pharmaceutical drugs in various dosage forms. By using TLC, many samples can be analyzed simultaneously and quickly with relatively low cost. Multiple separation techniques and detection procedures can be applied and the detection limits are often in the low nanogram range and quantitative densitometric methods are accurate. Moreover, TLC can be applied for the enantiomeric separation and analysis. To date, the issue of drug stereochemistry is of interest since the efficacy and/or safety of many drugs depend significantly on enantiomeric form whereas only 20 % of the optically active pharmaceuticals are sold as pure enantiomer. Consequently, efficient analytical procedures for control of optical purity are needed to supply modern procedures for asymmetric synthesis and TLC are always the analysis of choice.

Over the past decade, electrospinning has been gained rising popularity as a means of fabricating scaffolds with micro to nanoscale features. The advantages of electrospun fibers, nanoscale fibers, high uniformity, high surface-to-volume ratio, large porosity, and flexibility to fabricate a variety of 3D conformations, make them superior to those generated by other available techniques (Teo et al. 2006 : 89). It is now possible to produce a low-cost, high-value, high-strength fiber from a biodegradable and renewable waste product for easing environmental concerns. For

example, electrospun nanofibers can be used in wound dressings, filtration applications, bone tissue engineering, catalyst supports, non-woven fabrics, reinforced fibres, support for enzymes, drug delivery systems, fuel cells, conducting polymers and composites, photonics, medicine, pharmacy, fibre mats. From the reason aforementioned, electrospun fibers should also be suitable for application in the sorbent of thin layer chromatography.

In this research, nanofiber plate of cellulose acetate was prepared by electrospining technique and their capability for the separation using binary solvents containing methanol- water in various ratios were studied. In addition, the possibility of elelectrospun fiber plate for enantiomer separation was investigated.

CHAPTER 2
LITERATURE REVIEW

1. Introduction to thin layer chromatography

Thin layer chromatography is a kind of planar chromatography. It is the simplest of all the widely used chromatographic methods to perform. A suitable closed vessel containing solvent and a coated plate are all that are required to carry out the separations. With optimization of techniques and materials and the use of available commercial instruments, highly efficient separations and accurate and precise quantification can be achieved. TLC can also be used for preparative-scale separations by employing specialized layers, apparatus, and techniques.

Basic TLC is carried out as follows. A small aliquot of sample is placed near one end of the stationary phase, a thin layer of sorbent, to form the initial zone. The sample is then dried. The end of the stationary phase with the initial zone is placed into the mobile phase, usually a mixture of two to four pure solvents, inside a closed chamber. If the layer and mobile phase were chosen correctly, the components of the mixture migrate at different rates during movement of the mobile phase through the stationary phase. When the mobile phase has moved an appropriate distance, the stationary phase is removed, the mobile phase is rapidly dried, and the zones are detected in daylight or under ultraviolet (UV) light with or without the application of a suitable visualization reagent.

Differential migration is the result of varying degrees of affinity of the mixture components for the stationary and mobile phases. Various separation mechanisms are involved, the predominant forces depending upon the exact properties of the two phases and the solutes. The interactions involved in determining chromatographic retention and selectivity include hydrogen bonding, electron- pair donor/electron-pair acceptor (charge transfer), ion-ion, ion-dipole, and van der Waals interactions. Among the latter are dipole-dipole, dipole-induced dipole, and instantaneous dipole-induced dipole (London) interactions.

Sample collection, preservation, and purification are problems common to TLC and all other chromatographic methods. For complex samples, the TLC

development will usually not completely resolve the analyte from interferences unless a prior purification (cleanup) is carried out. This is most often done by selective extraction and column chromatography. In some cases substances are converted, prior to TLC, to a derivative that is more suitable for separation, detection, and/or quantification than the parent compound. TLC can cope with highly contaminated samples, and the entire chromatogram can be evaluated, reducing the degree of cleanup required and saving time and expense. The presence of strongly adsorbed impurities or even particles is of no concern, because the plate is used only once.

Detection is simplest when the compounds of interest are naturally colored or fluorescent or absorb UV light. However, application of a detection reagent by spraying or dipping is required to produce color or fluorescence for most compounds. Absorption of UV light is common for most aromatic and conjugated compounds and some unsaturated compounds. These compounds can be detected simply by inspection under 254 nm UV light on layers impregnated with a fluorescence indicator (fluorescence quench detection).

Compound identification in TLC is based initially on a comparison of Rf values to authentic reference standards. Rf values are generally not exactly reproducible from laboratory to laboratory or even in different runs in the same laboratory, so they should be considered mainly as guides to relative migration distances and sequences. Factors causing Rf values to vary include dimensions and type of chamber, nature and size of the layer, direction of the mobile-phase flow, volume and composition of the mobile phase, equilibration conditions, humidity, and sample preparation methods preceding TLC. Confirmation of identification can be obtained by scraping the layer and eluting the analyte followed by infrared (IR) spectrometry, nuclear magnetic resonance (NMR) spectrometry, mass spectrometry (MS), or other spectrometric methods if sufficient compound is available.

In the aspect of quantitative TLC, three approaches can be used: extraction of the spot with subsequent measurement by spectroscopic or other techniques, comparative separations with visual assessment and finally optical scanning. The first is rarely used due to the difficulties involved in extracting the material from the spot, which often gives poor accuracy.

For the comparative spot assessment by visual estimation, the sample is run in parallel with a series of calibration solutions each containing the solute of interest at different concentrations. The first set of calibration standards can have relatively large concentration intervals, which allows the approximate concentration of the solute of interest to be identified. A second set of standards is then made up that embraces the suspected concentration in smaller intervals and the separation is repeated. By matching the intensity of the spots, the concentration of the sample can be estimated.

The TLC spots cannot be rendered visible by a destructive process (e.g., concentrated sulfuric acid) as such processes are not quantitative. If the solute of interest is colorless, a suitable colored derivative must be formed either before separation or more commonly after the separation by treating the plate with an appropriate reagent. Providing the separation is clean and a suitable colored derivative is employed, the visual comparative procedure can give quantitative results with an accuracy of about +/-10 %. Today, such accuracy is generally considered inadequate and as a result, the visual comparative technique has been largely replaced by instrumental methods of spot density measurement.

2. Measures of chromatographic system efficiency

2.1 Model of theoretical plates

The model of theoretical plates originates from the theory of distillation. It was adapted to chromatography in the pioneer work on the physicochemical foundations of this method accomplished by Martin and Synge (Martin and Synge 1941 : 1358; Martin and James 1952 : 679). The utility of this model in the highly sophisticated column techniques, e.g., gas or high-performance liquid chromatography, is long and indisputably recognized. The demand for the concept of theoretical plates in thin-layer chromatography seemed less in proportion to the comparatively lower separation efficiency of this method. In view of the recent and successful attempts to enhance efficiency in this field also, the idea of theoretical plates applied to thin-layer chromatography for the first time became actually and

fully relevant. Broadening of a chromatographic spot can be simply expressed in terms of the theoretical plate number N of the given chromatographic system:

$$N = 16 \left(\frac{lZ}{w} \right) \quad (1)$$

where l and z are the migration lengths of the mobile phase and solute, respectively, and w is the chromatographic spot width in the direction of the mobile-phase migration (see Fig. 1).

Although the values of N attained for different solutes on the same chromatographic plate proved to coincide fairly well, they usually differ significantly from the analogous values characteristic of another plate type. For this reason, the quantity N can be regarded as an approximate measure of the separating efficiency of chromatographic plates. It is proportional to the migration length of the mobile phase l, so that, the Z/w ratio being constant, an increase in l results in an increase of N and better separation. This proportionality of N and l is given by the relationship

$$N = \frac{l}{H} \quad (2)$$

where H is the so-called HETP value (i.e., height equivalent of a theoretical plate). The quantity H, or simply the plate height, measures the efficiency of a given chromatographic system per unit length of the migration distance, l, of the mobile phase. Small H values mean more efficient chromatographic systems and larger N values. The main goal of efforts to enhance performance of thin layers is the attainment of small H values and maximum N values. As in other chromatographic techniques, the efficiency of a given TLC system is better (i.e., H is smaller) for

1. Smaller particles of stationary phases or supports
2. Lower mobile-phase flow rates
3. Less viscous mobile phase
4. Smaller solute molecules

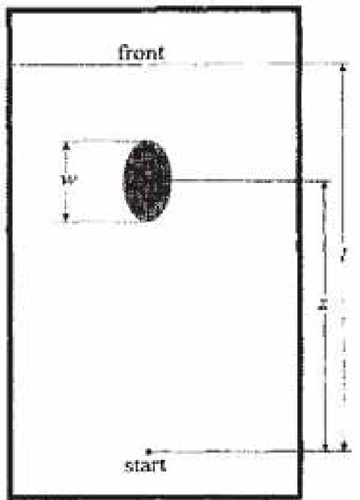


Figure 1 The thin layer chromatographic parameters used in calculation of the theoretical plate number *N*.

2.2 Van deemter equation

In the preceding subsection the simplest measure of spot broadening was introduced in the form of the quantity *H*, the plate height. One of the most important chromatographic relationships, the Van Deemter equation, attempts to estimate the relative contributions of eddy and molecular diffusion, and of the effects of mass transfer, on *H*. It is an empirical equation, originally established for column chromatographic techniques but valid also for thin-layer chromatography. The Van-Deemter relationship can be written in the complete version,

$$H = Au^{0.33} + \frac{B}{u} + Cu + Du \qquad (3a)$$

or simplified,

$$H = \mathrm{A}u^{0.33} + \frac{B}{u} + Cu \qquad \text{for } D = 0 \qquad (3b)$$

where u is the flow rate of the mobile phase and *A*, *B*, *C*, and *D* are the equation constants, measuring contributions of the different spot-broadening processes to the quantity *H*. The effects of eddy diffusion and mass transfer on the

flowing mobile phase are described jointly by *A*. The molecular diffusion is reflected in *B*, while *C* and *D* correspond to the effects of mass transfer in the stagnant mobile and stationary phases, respectively. The constants *A*, *B*, *C*, and *D* depend mostly on the parameters of the microporous solid, but they are also influenced by the nature of the solute and the mobile phase and by the working temperature of the chromatographic system.

Each constant of Eq. 3 can be defined as a function of certain properties of the chromatographic system. Let us briefly review the appropriate empirical relationships.

Giddings (Giddings 1965 : 323) proposed the following expression for *A*:

$$A = 2\lambda d_p \qquad (4)$$

where d_p is the diameter of a solid particle and *A* depends on the microscopic arrangement of solid bed.

B is given as

$$B = 2\gamma D_m \qquad (5)$$

where D_m is the diffusion coefficient of the solute in the mobile phase and γ is a correction factor mirroring the nonlinearity of diffusion due to the labyrinthine arrangement of micropores.

C is described by the equation

$$C = \frac{\omega d_p^2}{D_m} \qquad (6)$$

where ω is a proportionality factor. Similar to γ in Eq. 5, it also depends on the labyrinthine arrangement of micropores.

D is described by the relationship

$$D = \frac{\sigma d_f^2}{Ds} \qquad (7)$$

where d_f is the thickness of the stationary-phase layer, *Ds*, is the diffusion coefficient of the solute in the stationary phase, and a is a proportionality factor.

2.3 Separation and resolution

The R_f coefficient is the basic quantity used to express the position of solute on the developed chromatogram. It is calculated as the ratio

$$R_f = \frac{\text{Distance moved by the solute}}{\text{Distance moved by mobile phase front}} \qquad (8)$$

Using symbols from figure 1, R_f can be given as

$$R_f = \frac{Z}{l} \qquad (9)$$

R_f values are between 0 (solute remains on start) and 1.0 (solute migrates with front of mobile phase).

The traditional (and so far the only) method of determining the numerical values of analyte R_f coefficients quasi-automatically assumes the following preconditions:

1. Circular (or ellipsoidal) chromatographic band shape
2. Gaussian distribution of the mass of the analyte in this band

On the basis of these assumptions, the position of a band on the chromatogram is defined by measuring the distance between the origin and the geometrical center of the band. Despite the considerable imprecision of this definition for asymmetrical (i.e., tailing) and non-Gaussian bands, two features of the definition are very important:

1. The traditional definition regards the center of a chromatographic band as the point at which the local concentration of the analyte is the highest.

2. The traditional definition also regards the center of the chromatographic band as the center of gravity of the mass distribution of the analyte in the band.

For ideal, circular bands with Gaussian analyte concentration profiles, the band centers described by assumptions 1 and 2 are, in fact, identical.

For densitograms obtained from noncircular (i.e., tailing) bands with non-Gaussian concentration profiles, it can be stated that the numerical value of the R_f coefficient for a given chromatographic band can be determined for the maximum

value of the concentration profile of the band (which is the point at which the local concentration of the analyte is the highest). The R_f coefficient determined according to this definition can be denoted as $R_{f\,(\mathrm{max})}$.

Alternatively, the numerical value of the R_f coefficient can be determined from the center of gravity of the distribution of analyte mass in the band. With nonsymmetrical chromatographic bands, this value cannot be identical with that obtained from the maximum of the analyte concentration profile. The R_f coefficient determined in this second manner can be denoted as $R_{f\,(\mathrm{int})}$.

The main goal of chromatography is separation of a given solute mixture. However, it can happen that the chromatographic spots of two adjacent solutes overlap to a smaller or greater degree. Therefore, a demand arises for a measure of their separation. This demand is fulfilled by introduction of the quantity R_s, called resolution. The R_s of two adjacent chromatographic spots 1 and 2 is defined as being equal to the distance between the two spot centers divided by the mean spot widths (Fig. 2):

$$R_s = \frac{Z_2 - Z_1}{0.5(w_1 + w_2)} \qquad (10)$$

The quantity R_s serves to define separation. When $R_s = 1$, the two spots are reasonably well separated. Rs values larger than 1 mean better separation, and those smaller than 1, poorer separation.

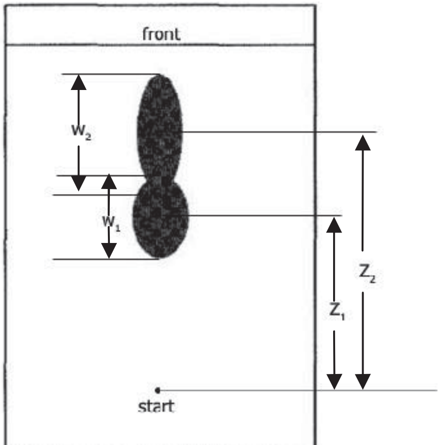


Figure 2 Illustration of resolution in thin-layer chromatography.

3. Sorbents

3.1 Sorbent selection

There are at least 25 inert materials that are available as sorbents in TLC, some of which have been more widely used than others. A number of the more important ones will be reviewed in this topic. Clearly for optimum separations, it is important that the correct material is chosen. Some sorbents have a specific range of application (e.g. silica gel impregnated with caffeine for polyaromatic hydrocarbons, or silica gel impregnated with a chiral selector for the separation of enantiomers of amino-acids and derivatives). By contrast silica gel or aluminium oxide is used for a wide range of applications. Silica gels and aluminas can also be split into a number of distinct, separate sorbents depending on pore size, particle size, and pH. Before choosing the sorbent, consideration must be given to the compounds to be separated. Characteristics, such as the polarity, solubility, ionisability, molecular weight, shape and size of the analytes are all important in deciding on a separation mechanism, and hence largely define both the type of sorbent and the solvents used both for the preparation of the sample and in development.

In 1973 Scott examined over 1100 papers to determine which sorbents were the most regularly used in TLC (Scott 1973 : 129). Silica gel was by far the most popular (~ 64%), followed by cellulose (~ 9%), and alumina (~ 3%). Since then silica gel has remained the most widely used, but noticeable changes have occurred with the appearance of chemically bonded phases which have opened up a new range of separation possibilities. The newer stationary phases have tended for the most part to address specific areas of separation where either the resolution of sample components was poor or non-existent. As the lists of applications for some sorbents is extensive, it is better to refer to the excellent bibliographies or abstract services that are available for TLC (e.g. Camag Bibliography Servicea) when a specific method from the literature is required. If this is not available to the user or a new or improved procedure is needed, then the basic information in Table 1 will be of help to ensure that the optimum sorbent for the type of separation is chosen.

Table 1 Choice of optimum TLC/HPTLC sorbents for compounds and compound classes

Sorbent	Compounds separated
Silica gel	All classes of compounds.
Aluminium oxide	Basic compounds (alkaloids, amines, etc.), steroids, terpenes, aromatic and aliphatic hydrocarbons.
Cellulose	Amino-acids and derivatives, food dyes (acidic and basic), carbohydrates.
Kieselguhr	Carbohydrates, aflatoxins, herbicides, tetracyclines.
Polyamide	Phenols, flavonoids, nitro-compounds.
Amino-bonded silica gel	Particularly good for carbohydrates, sulfonic acids, phenols, carboxylic acids, nucleotides, nucleosides.
Cyano-bonded silica gel	Many classes of compounds, particularly good for pesticides, steroids, preservatives.
Diol-bonded silica gel	Many classes of compounds, particularly good for steroids, hormones.
Reversed-phase (RP 2, RP 8, RP 18) silica gel	Improves separation for many classes of compounds (cf. silica gel) – steroids, tetracyclines, phthalates, antioxidants, lipids, barbiturates, capsaicins, aminophenols, fatty acids.
Chiral (CHIR) modified silica gel	Enantiomers of amino-acids, halogenated, N-alkyl, and a -methyl amino-acids, simple peptides, a -hydroxycarboxylic acids (catecholamines).
Silica gel impregnated with silver nitrate	Lipids, including variations in unsaturation and geometric isomers.
Silica gel impregnated with caffeine	Particularly selective for polyaromatic hydrocarbons.
Silica gel impregnated with boric acid/phosphate	Particularly selective for carbohydrates.

3.2 Silica based sorbents

Silica gel is by far the most frequently used layer material for adsorption TLC. Separations take place primarily by hydrogen bonding or dipole interaction with surface silanol groups by using lipophilic mobile phases, and analytes are separated into groups according to their polarity. Typical properties of TLC silica gel are a silanol group level of approximately 8 $\mu mol/m^2$; pore diameter of 40, 60, 80, or 100 A^o and specific pore volumes of 0.5-2.0 mL (Lepri and Cincinelli 2001 : 854). Specific differences in the types and distributions of silanol groups for individual sorbents may result in selectivity differences, and separations will not be exactly reproducible on different brands of silica gel layers (Bariska et al. 1999 : 46). Silica gel TLC plates are extremely versatile over a wide range of applications. Solvent mixtures composed of non-polar (e.g. hexane or cyclohexane) and polar (e.g. methanol, acetonitrile or water) constituents can be used without the chromatographic layer or binder being affected. Often acid modifiers (e.g. acetic, propionic or formic acids) or base modifiers (e.g. ammonia solution, pyridine or amines) are incorporated into the developing solvent to improve resolution.

3.3 Silica gel bonded phases

Bonded phases with functional groups chemically bonded to silica gel eliminate stripping of the stationary liquid from the support by incompatible mobile phases. Alkylsiloxane-bonded silica gel with CH_3, C_2H_5, C_8H_{17}, and $C_{18}H_{37}$ functional groups are most widely used for RP-TLC of organic compounds (polar and nonpolar homologous compounds and aromatics), weak acids and bases after ion suppression with buffered mobile phases, and strong acids and bases using ion-pair reagents. Layers from different companies but with the same bonded group can have different percentages of carbon loading and give different results. The hydrophobic nature of the layer increases with both the chain length and the degree of loading of the groups. Alkylsiloxanebonded layers with a high level of surface modification are incompatible with highly aqueous mobile phases and are used mainly for normal-phase separations of low-polarity compounds. Problems of wettability and lack of migration of mobile phases with high proportions of water have been solved by adding 3% NaCl to the mobile phase (Whatman layers) or preparing "waterwettable"

layers with a slightly larger particle size, less exhaustive surface bonding, and a modified binder. The latter layers with a low degree of surface coverage and more residual silanol groups exhibit partially hydrophilic as well as hydrophobic character and can be used for reversed-phase and normal-phase TLC. Chemically bonded phenyl layers are also classified as reversed-phase, but their use has only seldom been reported in the literature.

Hydrophilic bonded silica gel containing cyano, amino, or diol groups bonded to silica gel through a trimethylene chain [—$(CH_2)_3$—] are compatible with aqueous mobile phases and exhibit multimodal mechanisms. Polarity varies as follows: unbonded silica > diol-silica > amino-silica > cyano-silica > reversed-phase materials. Cyano layers can act as a normal or reversed phase, depending on the characteristics of the mobile phase, with properties similar to a low-capacity silica gel and a short-chain alkylsiloxane bonded layer, respectively. Amino layers are used in NP and weak anion-exchange modes. In NP-TLC, compounds are retained on amino layers by hydrogen bonding as with silica gel, but the selectivity is different. Charged substances such as nucleotides or sulfonic acids can be separated by ion exchange using

3.4 Cellulose

Cellulose, a product of natural origin, has a polymeric structure consisting of glucopyranose units joined together by oxygen bridges. As shown in Figure 3, a profusion of hydroxyl groups are present which are readily available for hydrogen-bonding. Adsorbed water or alcohols can be retained by this interaction, making cellulose an ideal phase for the separation of hydrophilic substances such as amino-acids, carbohydrates, inorganic ions and nucleic acid derivatives. Two types of cellulose are used in planar chromatography. One is native fibres with a typical polymerisation of between 400-500 glucopyranose units, (used for paper chromatography and in some TLC layers). The other is a microcrystalline form commonly called ‘Avicel’, a fine powder used widely in both TLC and HPTLC, and prepared by a hydrolysis technique. It has a degree of polymerisation of 40-200 glucopyranose units. The cellulose is obtained from a number of raw materials, including wood and cotton. However, the former does require more refining and has a

lower cellulose content. For the preparation of TLC/HPTLC plates, a similar slurry technique to that needed for silica gel is employed. However, unlike silica gel, binders are unnecessary. The chromatographic results obtained with either the fibrous or microcrystalline types can be different. However, whatever the type, the resolution of samples is generally not as sharply defined as that obtained with silica gel. Spots and bands are more diffuse and separation times are usually longer. Pre-coated plates are available from most of the TLC plate suppliers, but few supply a high-performance layer. The diffusion of chromatographic zones is greatly reduced with HPTLC cellulose, but one must remember to adhere strictly to the application of small quantities (~100 ng) of sample with final spot diameters of approximately 1 mm. Commercial pre-coated cellulose plates are usually made as thinner layers than for silica gel, nominally 0.1 mm thick.

Figure 3 Structure of cellulose illustrating the hydrogen-bonding effect with water

3.5 Cellulose bonded phases

PEI Cellulose. PEI cellulose is a polyethyleneimine modified cellulose which acts as a strongly basic anion-exchanger. It has had fairly specific uses including the analysis of nucleotides, nucleosides and nucleo-bases, vanadylmandelic acid (VMA), and sugar phosphates (Randerath 1963 : 195). For these applications PEI cellulose was the sorbent of choice for many years. Most of these separations are now achievable on amino-bonded silica gel layers with improved resolution. The PEI cellulose TLC layers require storage at 0–4 ^{o}C to reduce deterioration. As the plates become old, the layers take on a pale brown coloration, and should be discarded.

Acetylated cellulose (CA), particularly triacetyl-cellulose, is prepared by chemical reaction of the hydroxyl groups on cellulose to produce a layer with reversed-phase characteristics. Its major use for many years has been for the separation of polyaromatic hydrocarbons, an important area of interest. However, in many cases it has been superseded by bonded reversed-phase silica gels. In more recent times the use of acetylated cellulose as a chiral layer for the separation of optical isomers has been investigated (Lepri 1994 : 376). The resolution of enantiomers is very dependent on the cellulose structure and the acetyl content of the cellulose triacetate. The best results are obtained with a layer of microcrystalline cellulose triacetate (particle size of 10 µm) with a silica gel 60 binder. Although the sodium salt of carboxymethylcellulose has also been used as binder, silica gel enables the use of aqueous based eluents. As with column separations of enantiomers, mixtures of ethanol or propan-2-ol (70–80%) with water (20–30%) serve well as mobile phases. Resolution varies according to organic solvent concentration in the eluent. It has also been observed that temperature has a noticeable effect on the quality of separation. Generally as temperature increases from 25 to 40 ^{o}C, resolution of enantiomers decreases. Racemates of a number of organic species have been separated on TLC layers of microcrystalline cellulose triacetate. These include specific compounds such as benzoin, benzoin methyl ether, flurbiprofen, 1-(2-naphthyl) ethanol, aminoglutethimide, 1,1'-binaphthyl-2,2'-diamine, N-[1-(naphthyl) ethyl] phthalamic acid, and a few derivatised amino-acids. However, to date the use of this chromatographic layer has not developed commercially probably due to the long

development times that are required (about 2.5 hours) for sufficient migration of analytes.

Carboxymethyl (CM) and diethylaminoethyl celluloses (DEAE) are prepared as ion-exchange media, the former is weakly acidic and the latter is strongly basic. The exchange capacities are often close to those for ion-exchange resins, but their behaviour is often quite different. This is due to the hydrophilic nature of the base cellulose compared with the hydrophobic nature of the base polymer in the resin material.

3.6 Aluminium oxide

Aluminium oxide or alumina, like silica gel, is a synthetic sorbent. It is manufactured in three pH ranges; acidic, basic, and neutral for different types of samples. Thus under aqueous conditions acidic compounds like phenols, sulphonic, carboxylic, and amino acids are separated on the acidic alumina, whilst basic compounds; amines, imines, and basic dyes, are separated on basic alumina. Neutral compounds, such as aldehydes, ketones and lactones are chromatographed on neutral alumina. Of the three types, basic alumina is the most widely used. In non-aqueous eluents, aromatic hydrocarbons, carotenoids, porphorins, alkaloids, and steroids can be adsorbed. As with silica gel, alumina will also vary in activity according to water content.

3.7 Kieselguhr

Kieselguhr is a natural diatomaceous earth, composed of the skeletal remains of microscopic marine organisms deposited in times past. Although principally silicon dioxide, it also contains varying amounts of other oxides of aluminium, iron, titanium, magnesium, sodium, potassium and calcium as oxides, hydroxides, and carbonates (approximately 10% in all).(Rössler 1969 : 28) It is widely used as a filter aid due to its high porosity (average pore diameter is quite variable, typically 65000 A˚). Kieselguhr is used in conjunction with 15% of a calcium sulphate binder to produce TLC plates. The variability of pore size and surface area limits the use of kieselguhr for high quality, precision TLC. It has been used in the past for the separation of polar compounds by a partition mechanism. Commercial pre-coated plates with abrasion

resistant organic binders have been available for many years, although their usage has diminished in recent times.

3.8 Polyamide

The polyamide phases are produced from polycaprolactam (nylon 6), polyhexamethyldiaminoadipate (nylon 66), or polyaminoundecanoic acid (nylon 11). The chromatographic separation on polyamide depends on the hydrogen-bonding capabilities of its amide and carbonyl groups (Figure 4). The bond strength generated depends upon the number and position of any phenolic, hydroxyl or carboxyl groups present in the sample components. The relative retention of the analytes depends on the eluting solvent being capable of dissociating these bonds. As the solvent migrates through the sorbent, the analytes separate according to their ease of displacement. Mixtures of phenols, indoles, steroids, nucleic acid bases, nucleosides, dinitrosulfonyl (DNS), dinitrophenyl (DNP), and dimethylaminoazobenzene isothiocyanate (DABITC) derivatised amino-acids, and aromatic nitro compounds have all been resolved on polyamide (Bushan 1991 : 353; Soczewin and Szumilo 1973 : 99–107). A range of pre-coated sheets with aluminium or plastic backing are commercially available, including one quite unique 15 cm square plastic sheet which is coated on both sides with polyamide 6. With this plate, samples containing, for example, amino-acid derivatives are applied to one side whilst the standards are put on the other. After chromatography the known aminoacids can be picked out immediately. This novel approach has been successfully applied to PTH, dansyl, and DNP amino-acids.

Figure 4 Hydrogen-bonding of water with polyamide (nylon 66).Compounds that will form stronger hydrogen-bonds will require a stronger elutive solvent to cause migration

3.9 Mixed stationary phases

Mixed phases, for example silica gel/kieselguhr, silica gel/alumina and cellulose/silica gel are sometimes used for specific applications. However, they almost always require the preparation of a special layer with a specific ratio of components. Few commercial pre-coated plates are available. Silica gel/kieselguhr has been used for inorganic ions, herbicides and some steroids (Gregorowicz 1971 : 169). Cellulose/silica gel has found application in the separation of food preservatives, and antibiotics (König and Schüller 1979 : 36.). Silica gel/alumina has been little used in the last twenty years.

3.10 Miscellaneous stationary phases

Other less commonly used phases include magnesium silicate, chitin and Sephadex^{TM}. Magnesium silicate is a white, hard powder often known under the name of FlorisilTM. The manufacturer, Floridin, (Pittsburgh, USA) gives the surface area of the TLC grade as 298 m^2 g^{-1} and the pH as 8.5. It has been reported as suitable for the separation of carbohydrates and derivatives.

Chitin is a polysaccharide composed mainly of 2-acetamide-2-deoxy-D-glucan molecules linked via oxygen bridges in a similar type of structure to cellulose but with a basic nature. Typical specific surface area is low, only 6 m^2 g^{-1}. Chitin has been used principally for amino-acid separations, but it has also been applied to inorganic ions, nucleic acids, phenols and dyes. Sephadex is a trade name of Pharmacia Fine Chemicals for a range of gel filtration materials. They are modified dextrin gels, hydrophilic and neutral in nature. They are rarely used in TLC as layers are difficult to prepare, and require pre-swelling for many hours before use. Sephadex has been used for the separation of peptides and nucleic acids.

4. Preparation of TLC sheets and plates

4.1 ''Home made'' TLC plates

Before the advent of commercial pre-coated TLC plates, layers of silica gel and of other sorbents (e.g. cellulose or aluminium oxide) had to be prepared in advance of separation procedures. A typical method was to thoroughly mix 30 g of sorbent with 60 mL of water by shaking in a glass flask. The slurry produced was transferred to a spreader and applied evenly over the plate surface in one spreading operation. The applied slurry was allowed to set and dry for about 30 min. Final activation was completed in an oven for 30 min at a maximum temperature of 105 ^{o}C. To improve the binding of the silica gel to the inert backing a gypsum binder was added; Stahl recommended 13% of calcium sulphate. Although the layer was soft and easily damaged, it remained stuck to the glass backing when the mobile phase migrated across the plate. This silica gel plate was given the designation ''silica gel G''. Organic binders have also been used including carboxymethylcellulose, starch (1–2%), and polyvinyl alcohol (1–5%). Although these give a stronger binding for the most part at a lower concentration of binder, they often suffer from solubilisation in aqueous based solvents and charring after treatment with strong sulphuric acid solutions and heating.

4.2 Pre-coated TLC/HPTLC sheets and plates

Commercially manufactured pre-coated TLC and HPTLC plates use organic polymeric binders at a concentration of about 1–2%. These binders are much more resistant to chemical elution or attack, ensure a smooth abrasive resistant surface, and effectively bind smaller particle size silica gels to an appropriate backing such as glass, aluminium or plastic. Quite strong polar solvents and detection reagents can be used without appreciable affect. The added advantage is that the plates provide a layer of highly active silica gel that is not deactivated by appreciable concentrations of inorganic binders. There is always a noticeable improvement in the quality of separation between such plates and those consisting of silica gel G. Most TLC and HPTLC silica gel plates are manufactured with a uniform surface and a layer thickness of 0.20–0.25 mm (the major exception is cellulose with a layer thickness of 0.1 mm). These commercially manufactured plates give far more reproducible results

compared with those that are ‘‘home-made’’. This is a major benefit in analytical work where repeatable quantitative results are essential. Particle size, pore diameter, smoothness and thickness of surface, and abrasive resistant qualities of a layer need to be controlled within fine tolerances by the manufacturer to ensure such high quality and reproducible results.

5. TLC in pharmaceutical analysis

Although HPLC has superseded TLC for many applications. TLC in both instrumental or noninstrumental forms has remained standard method for solving many difficult analytical problems. The most important tasks of capillary action planar chromatography in pharmaceutical analysis are summarized in Table 2.

Because of its useful role when cost effectiveness is essential, TLC is widely used as the standard technique for rapid and accurate identification of raw materials or finished products as well as for purity testing of raw materials and formulations in various pharmacopoeial prescriptions. The importance of TLC for assay methods, considering the difficulties experienced during its use, has decreased, and only a few official methods can be found in the pharmacopoeias, usually with a spot elution technique for quantification. In the field of industrial pharmaceutical analysis, the situation is different, because TLC instrumentation has reached a relatively high level of sophistication. In some special application areas, such as the analysis of extracts of medicinal plants and fermentation mixures, modern TLC (precoated or HPTLC layers, densitometric evaluations) has a district role, because the interference of “unknown background materials” can be more easily eliminated than with other chromatographic techniques. Many chromatographers working in pharmaceutical industry prefer to use reversed-phase HPLC in conjunction with normal-phase TLC or HPTLC to analyze raw meterials for purity and impurities as well as for stability testing.

Table 2 Application of capillary planar chromatography in pharmaceutical analysis

Properties	Advantage	Disadvantage
Type of solute		
Very polar		Limited
Polar	Good	
Medium polar	Good	
Nonpolar	Good	
Structure of solute		
Structurally different compounds	Good	
Isomers	Good	
Homologious compounds		Limited
Size of solute		
Small	Good	
Medium large		Limited
Large		Limited
Analysis of starting raw materials		
Plant extracts	Widely used	
Extracts of animal organs	Widely used	
Fermentation mixture	Widely used	
Analysis of intermediates		
Intermediates and crude products		Limited
Reaction mixtures	Applicable	
Mother liquors and secondary products	Applicable	
Analysis of pharmaceutical raw materials		
Identification	generally used	
Purity testing	Alternative to HPLC	
Assay		Not applicable
Stability testing	complementary to HPLC	
Analysis of formulated products		
Identification		
Purity testing	Generally used	
Assay	Alternative to HPLC	
Stability testing	complementary to HPLC	
Content uniformity test	complementary to HPLC	Limited
Dissolution test		Limited

5.1 Identification of known and unknown compounds

The basic test in each pharmacopeia is the identification of the compounds by TLC. In United States Pharmacopeia and European Pharmacopoeia, many monographs contain TLC methods; most of them are identify tests. The TLC is carried out by using standardized experimental conditions. Spot of the substance being examined with or without derivatization is compared to the spot of similarly developed reference material applied in the same concentration. The sizes and positions of the spots of the principal component and the reference standard must be similar.

The most case, different TLC systems are used for identification of substances rather than for purity testing. The reason is sample; in the first case the main aim of the analysis is to differentiate compounds belonging to the same type (group-type separation, separating compounds with similar but not the same structure), whereas in the second instance (purity testing), structurally related compounds can be separated.

When synthesizing a new compound, various chromatographic techniques are used to discover the impurity profile of the new substance. In this case, in addition to HPLC and GC, TLC methods have importance roles (Gorog et al. 1998 : 511).

The identification of unknown compounds is difficult analytical task. The use of retention data alone is not sufficient for this purpose, because of the high risk of coelution of compound in question with many other structurally similar or different compounds in the same chromatographic system. Off-line combined techniques such as TLC-HPTLC, TLC-MS, and TLC-IR are frequently applied for structure elucidation and identification of an unknown compound in the chromatogram.

5.2 Purity Testing

Many countries require impurities in bulk raw material should be below 1%, and any individual impurity present at concentration above 0.1% must be identified. The official methods (pharmacopoeial methods) in this regard are different from the methods use in industrial analysis : HPLC is the premier method, and TLC is the second choice. Pharmacopoeias are in agreement that conventional TLC is used

without instrumentation. In Industrial pharmaceutical analysis, HPLC and instrumental TLC are more or less equally used, in many case in conjunction. It should be noted that TLC has been revitalized by the recent availability of new bonded phases and by better instruments for development and quantification.

TLC is usually involved in the estimation of an impurity profile. Several methods have been published that use instrumental TLC for stability testing of active ingredients and formulated products. However, if the impurities cannot be identified with the aid of authentic reference standards of the supposed impurities, then spectroscopic investigation of separated impurities is necessary.

5.3 Assay methods

Assays are expected to be accurate (giving the true contents of analytes in the mixture), selective (being able to distinguish between analytes and related compounds), and relatively simple. These guideline explain the general preference for chromatographic methods. As was mentioned, HPLC superseded TLC methods in the case of active pharmaceutical ingredients from this analytical field owing to the problems and difficulties related to quantification in TLC.

In the case of formulated products, the densitometric TLC/HPTLC assay method is frequently used. Nearly 200 examples of this type of analysis are reported in the book by sethi (Sethi 1996). These methods provide fast and precise results.

TLC densitometric methods are also suitable for testing the content uniformity of tablets. For H_2 receptor antagonist pharmaceuticals using the eluent toluene-methanol-diethylamine (9:1:1, v/v) (Novakovic 1999 : 193).

5.4 Standardization possibilities

Official methods described in various pharmacopoeias can be considered standardized methods. Among these methods, some general ones are worthy of mention and are listed in Table 3.

Table 3 Methods Used for Group-Type Separation in USP and Ph. Eur

Analytical aim	stationary phase	Mobile phase	Detection	Literature source
Identification of fatty oils	HPTLC octadecylsilyl silica gel	Methylene chloride-glacial acetic acid-	Spay with 10% phosphomolybdic	Ph. Eur. 4, 2.3.2
Identification of Phenotiazines	Kieselguuhr G impregnated with a mixture of 10% phenoxyethanol+ 50 g/L of Macrogol 300 in acetone	50 mL light petroleum, 1 mL diethyl-amine saturated with phenoxyethanol	In UV (366 nm) after spraying with 10% ethanolic sulfuric acid	Ph. Eur. 4, 2.3.3
Test for foreign oils	Kieselguuhr G impregnated with a mixture of 10 vol liquid paraffin and 90 vol petroleum spirit	water-glacial acetic acid (10:90)		Ph. Eur. 4, 2.4.22
Identification of Tetracyclines	Octylsilanized silica gel	0.5 M oxalic acid (previously adjuted with ammonium hydroxide to pH 2.0)-Acetonitrile-methanol (80:20:20)	Ammonia vapour, then UV, 366 nm	USP 25 (193)
Test for aflatoxin in Materials of plant origin	Silica gel	Chloroform-acetone-isopropyl alcohol (85:10:5)	UV, 366 nm	USP 25 (561)
Identification by TLC, general	Silica gel	Chloroform-methanol-water (180:15:1)	UV, 254 nm	USP 25 (201)

5.5 Thin-layer chromatography of steroids

Steroids are a class of compounds that have a cyclopentanoperhydrophe- nanthrene skeleton and that occur in nature and in synthetic products. The bile acids, androgens, estrogens, corticosteroids, sterols and vitamin D are compounds included in the class of steroids.

Steroids and their metabolites are analyzed by thin-layer chromatography (TLC) in a variety of samples such as biological samples or plants and pharmaceutical formulations. TLC continues to be an important method for the determination of steroids because of its advantages. Many samples can be analyzed simultaneously and quickly at relatively low cost, multiple separation techniques and detection procedures can be applied and the detection limits are often in the low nanogram range and quantitative densitometric methods are accurate. The importance of steroid analysis is evidenced by many papers and chapters of books (Szepesi et al. 1996 : 685; Mulja and Indrayanto 2001 : 794; Sherma 2003 : 913).

Many chromatographic systems have been applied for the TLC of steroids. Silica gel TLC and HPTLC layers are the most frequently used. C-8 and C-18 modified silica gel (Petrovic et al 2000 : 106), alumina (Acanski 1998 : 97), silica gel with 3–10% silver nitrate (Li et al. 1995 : 372), cellulose unimpregnated or impregnated with 1,2-propanediol (Mulja and Indrayanto 2001 : 794) and kieselgur (Sherma 2003 : 913) have been used as stationary phases.Most of the reagents used for detecting steroid spots contain sulfuric acid. Antimony trichloride, tetrazolium blue, molybdophosphoric acid, chlorosulphonic acid, acetic acid and phosphoric acid have been used as destructive reagents for steroids detection. Table 4 shows several thin layer chromatographic systems designed for the analysis of steroids.

Table 4 Thin layer chromatographic analysis of steroids

Analyte	Stationary phase	Mobile phase	Remarks
Cholestrol, allylestrenol, pregnanediol,etc.	RP-HPTLC plates	Acetronitrile/methanol, acetronitrile/water and methanol/water	Investigation of the retention behavior of 12 sreroids.Mixture of 10g copper sufate and 5 mL o-phosphoric acid (86%) dissolved in 95 mL methanol.
Hydrocortisone, prednisolone, mesylate, etc.	Silica	Benzene/ethylacetate (1:1)	Use of colour photodocumentation of UV irradiated thin layer chromatograms for the analysis of steroids.Detected by spraying with a 10% ethanolic solution of sulfuric acid followed by heatingat 100°C for 2 to 4 min
Steroids	Silica impregnated with silver nitrate	1.Hexane/ethylacetate (3:1,2:1)	Silver nitrate impregnated silica layers were used for the separation of steroids.
Steroids	NH_2 F245s	Chloroform/ethanol/formic acid (50:10:10)	Analysis and separation of steroids on NH_2 layers.
Estradiol, hydrocortisone, testosterone and cholesterol	Diol F254s	Chloroform	The densitometric detection of these compounds with and without the use of sulfuric acid solutions as visualizing reagents was compared.

Source : Bhawani, S.A. et al. “Thin-Layer Chromatographic Analysis of Steroids:A Review.” Tropical Journal of Pharmaceutical Research. 3, 9 (2010) : 301-331.

6. Thin-layer chromatography of enantiomers

Because of different biological activities on enantiomer of active ingredients. Frequently, only one of the two antipodes is pharmaceutically active, while the other may be at best inactive or even toxic. Only about 20% of the optically active pharmaceuticals are sold as pure enantiomers (Xuan 1995 : 382). This has resulted in an increasing interest in stereoselective syntheses based on chiral intermediates. The production of these so-called auxiliaries ultimately requires enantiomerically pure natural substances, with optically active amino acid playing an important part as a chiral pool. Consequently, efficient analytical procedures for control of optical purity are needed to supplement modern procedure for asymmetric synthesis.

Polarimetry is used in many laboratories for control of optical purity. However, calculation of the enantiomeric excess from optical rotation is often impossible because the specific rotation of the pure enantiomer is not known precisely, or calculated enatiomeric excess values may be incorrect owing to impurities. For these reasons direct chromatographic analytical procedures are preferred. Because simple separation techniques are known, gas chromatographic (Bhushan and Martens 2001 : 155) and high-pressure liquid chromatographic (Testa 1986 : 265; Nishi and Fujimura 1990 : 187) and capillary electrophoresis (Tanaka and Terabe 1997 : 151) procedures are generally used for direct determination of enantiomeric composition. These systems require costly equipment; sometimes sample derivatization is necessary; and for routine applications, standardized stationary phases must be commercially available.

Aplication of thin-layer chromatography (TLC) separation technique is desirable, especially with large test series. TLC may use one of three basic techniques for separation of enantiomeric compounds:

1. Separation on ordinary stationary phases by means of chiral additives in eluent, which form diastereomeric complex with the substrate.

2. Direct separation by using chiral stationary phases, effected by the formation of diastereomeric association complexs.

3. Separation on achiral stationary phase via diastereomeric derivatives formed by reaction of the sample with a chiral reagent

6.1 Enantiomeric separations on microcrystalline triacetylcellulose thin-layer plates

6.1.1 Resolution Mechanism

The resolving capability of this polysaccharide derivative is based on its morphological structure. Peracetylation of the cellulose has to be performed such that the conformation and relative position of the carbohydrate bands in their crystalline domains remain intact. In this state cellulose triacetate includes enantioselectivity; i.e., antipode separations are possible (Hesse 1976 : 62)

6.1.2 Survey of applications of racemic separations

In 1973 Hesse and Hagel (Hesse and Hagel 1973 : 277) for the first time described the thin layer chromatography racemate separation of Troeger's base on cellulose triacetate. These plates are stable with aqueous eluent systems resistant to dilute acids and bases. They are stable in alcoholic and phenolic eluents but are attacked by glacial acetic acid and ketonic solvents.

Günther and Merget (Lepri et al. 1994 : 376) were successful in separating the pesticide (±)-2-(4-chloro-6-methylamino-[1,3,5]-triazin-2-ylamino)-2-methylbutyronitrile on a microcrystalline triacetylcellulose plate. Further separations of microcrystalline triacetylcellulose plates were done by Lepri et al. (Lepri et al. 2000 : 384) and Wang (Wang et al. 1997 : 612)

6.2 Thin-layer enantiomeric resolution via ligand exchange

6.2.1 Resolution mechanism

Experimental results have confirmed the principle of chiral interaction (three-point rule) postulated in 1952 by Dalgliesh (Dalgliesh 1952 : 3940). Additionally, the result prove that the separation models developed for ligand exchange by high-performance liquid chromatography are also valid for TLC; the diastereomeric complexe formed with metal ion (e.g., Cu^{2+}) and the chiral adsorbent have different stabilities for the different antipodes, and thus chromatographic separation is achieved.

6.2.2 Survey of Applications of Racemic Separations

Thin layer chromatographic enantiomeric separations based on ligand exchange were published independently by Günther et al.(Günther et al. 1984 : 506) and Weinstein (Weinstein 1984 : 985). Though very similar in their technique, the procedures differ in their choice of chiral selector and consequently in their range of applicability. Using commercially available reversed-phase TLC plates, Weinstein (Weinstein and Grinberg 1984 : 251) impregnated the layers with the optically active copper complex of N,N-di-n-propyl-L-alanine after preconditioning the ready-to-use plate with buffer A (0.3 M sodium acetate in 40% acetonitrile and 60% water, adjusted to pH 7 with acetic acid). With the exception of proline, all proteinogenic amino acids were resolved –as dansyl derivatives– into L- and D-enantiomers. Another paper from this group (Marchelli et al. 1986 : 354) described a two-dimensional reversed-phase thin layer chromatographic procedure for simultaneous separation of racemic dansyl amino acids mixtures. In the first direction the dansyl amino acids were separated on RP-18 TLC plates with eluents without chiral additives using, e.g., a convex gradient with increasing acetonitrile content (20-30%) in 0.3 M sodium acetate (pH 6.3). In the second direction the plate was treated with that above-mentioned chiral selector and then again developed with aqueous acetonitrile – sodium acetate buffer. The separation was further improved by using a temperature gradient (6.2 ^{o}C/cm). The influence of the temperature on enantiomeric separation behavior. Chiral diaminodiamide copper (II) complexes are also suitable as chiral selectors for thin layer chromatographic enantiomeric separations of racemic dansyl amino acids (Davankov et al. 1980 : 677). In these ligands two L-amino acids are joined via an amide bond by ethylene and trimethylene bridges and are endowed with varying degrees of lipophilicity and bulkiness, depending on the nature of the amino acid side chain. The coating procedure in general corresponds to that of Weinstein. The authors also work with one- or two-dimensional techniques with or without chiral additive in the eluent (acetonitrile-water, 33:67, adjusted to pH 6.8 with acetic acid).

Based on the work of Davankov and coworkers, who modified commercial HPLC columns for distribution chromatography with alkyl derivatives of L-amino acids such as n-decyl-L-histidine or n-hexadecyl-L-proline, Günther et al. used (2S,4R,2'RS)-N-(2'-hydroxydodecyl)-4-hydroxyproline which is easier to

prepare, as a chiral selector. The following impregnation procedure proved to be most efficient. A glass plate coated with hydrophobic silica gel (RP-18 TLC) was dipped into a 0.25% of copper(II) acetate solution (methanol-water, 1:9) and dried. Then the plate was immersed in a 0.8% methanolic solution of the chiral selector for 1 min. after air drying, the plate was ready for enantiomeric separations. Unlike the procedures described above, in this case antipode separation of amino acids was possible without derivatization. Because the commercially available chiral TLC/HPTLC plates are based on this ligand-exchange chromatographic technique.

Efforts were made to illuminate the structure of the complex of the 4-hydroxyproline selector and to find new selectors for the enantiomeric separation based on ligand-exchange chromatography. Martens and coworkers (Martens et al. 1987 : DE-PS 3143726) tried to do X-ray investigations of the 4-hydroxyproline copper(II) complex, but it was not possible to get a crystalline complex of this selector. Therefore they synthesized a model compound with a methyl group in instead of the $C_{10}H_{21}$ group. With this short alkyl group-modified compound with a methyl group also mentioned that the configuration in the 2′-position of the side chain of the 4′-hydroxyproline selector has no influence on the stereoselectivity of its copper complex in the enantiomeric separation of amino acids (Martens et al. 1990 : 7127). Recently, new experiments on the crystallization and structure determination of the copper (II) complex were successful. The results show that coordination at the copper center of the selector complex is fundamentally different from that of the short alkyl chain model compound.

Other selectors for the separation of enantiomers based on ligand exchange chromatography were synthesized iminocarboxylic acid was used for the enantiomeric separation of 5,5-dimethyl-3-thiazoline-4-acetic acid with the eluent system acetonitrile-methanol-water (3:5:5), whereas Sinibaldi et al. (Sinibaldi et al. 1988 : 1245) resolved D,L-dansyl amino acids on reversed-phase TLC plate pretreated with a Cu^{2+} complex of poly-L-phenylalanine amide.The polymeric ligand was synthesized by the reaction of optically active amide with ethylene glycol diglycyl ether. The method makes use of a sophisticated liquid chromatograph for obtaining the desired polymer fraction, which is subsequently used for the LEC, and

this might limit the application of the separation procedure. However, a simple method was used by Bhushan et al. (Bhushan et al. 1994 : 126). Here L-proline was used as a chiral selector on normal-phase silica gel (Bhushan 1994 : 126), and amino acids were resolved with the eluent systems n-butanol-acetonitrile-water (6:2:3), chloroform-methanol-propionic acid (15:6:4), and acetonitrile-methanol-water (2:2:1). Another paper was reported by Bhushan et al., Atenolol and propranolol and salbutamol were resolved into their enantiomers by adopting different modes of loading/impregnating the Cu(II) complexes of L-proline (L-Pro), L-phenylalanine (L-Phe), L-histidine (L-His), N,N-dimethyl-L-phenylalanine (N,N-Me(2)-L-Phe), and L-tryptophan (L-Trp) on commercial precoated normal phase plates. (Bhushan et al. 2010 : 1395)

Until now these selectors showed no eminent advantage compared with the 4-hydroxyproline selector. Therefore, layers using the 4-hydroxyproline selector (Figure 5) are only commercially available ready-to-use plates (Chiralplate®, CHIR®).

Figure 5 Hydroxyproline

6.3 Separation of enantiomers using chiral *β*-cyclodextrin

6.3.1 Resolution mechanism

β-Cyclodextrin (*β*-CD) is a chiral, toroidal molecule consisting of seven glucose units connected via *α*-1,4 linkages (Figure 6). The enantiomers are selectively retained because they fit differently into the cavity of the oligomer.

6.3.2 Survey of Applications of Racemic Separations

Alak and Armstrong (Armstrong et al. 1990 : 65) investigated the influence of different silicas and binders on the separation behavior of *β*-cyclodextrin TLC plate. Besides nine racemates, three diastereomeric compounds and six structural

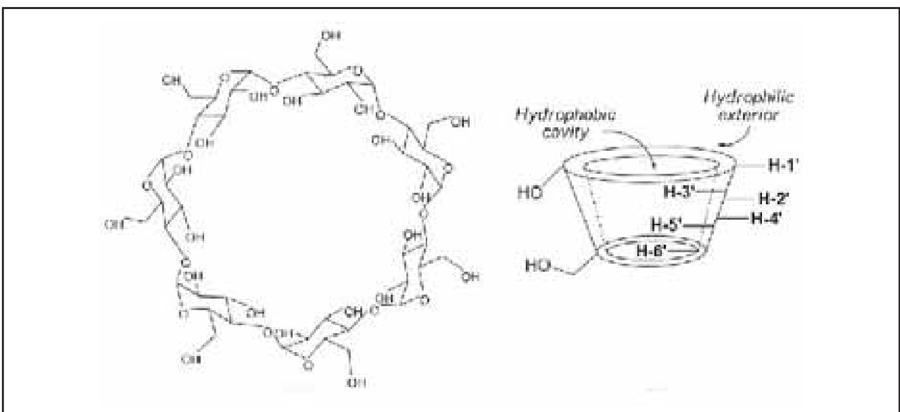


Figure 6 β-Cyclodextrin

isomers were separated. Wilson (Wilson 1986 : 277) impregnated silica plates with a 1% solution of β-cyclodextrin in ethanol-dimethylsulfoxide (80:20 vy volume); racemic mandelic acid was barely separated, and the antipode separation of β-blockers was not possible.

Bhushan and Martens (Bhushan and Martens 1997 : 280) presented a paper concerned with methods of impregnation of thin-layer materials with a variety of regents and the role of impregnation in enantiomeric separation. Armstrong et al. (Armstrong 1988 : 345) were the first to describe application of β-cyclodextrin as a chiral eluent additive for separations on reversed-phase TLC plates. The success of separation was strongly dependent on type and quatity of modifier applied but above al on the concentration of β-cyclodextrin. The low solubility of in water (0.017 M, 25 ^{o}C) can be improved by addition of urea; sodium chloride stabilizes the bider of the RP plates. Compared to β-cyclodextrin bonded phases, a reversed retention behavior was noticed, the D-enantiomer eluting above the L-isomer. The separation of steroid epimers and other diastereomeric classes of compounds is also possible with this technique. Hydroxypropyl and hydroxyethyl β-cyclodextrin are also suitable as chiral mobile-phase additives for thin layer chromatographic enantiomer separations (Gerald 2004 : 432). Their better solubility in water and aqueous-organic eluents (compare to β-cyclodextrin) enhances enantioselectivity; 0.6 M substituted β-cyclodextrin has proven especially active for

separation. Armstrong (Armstrong et al. 1988 : 323) plates using the mobile phase acetonitrile-water containing maltosyl- β-cyclodextrin. The preferred TLC plate was the ethyl-modified one because a greater number of compounds were separated using this type of plate.

Lepri (Lepri et al. 1990 : 311) and Duncan (Duncan and Armstrong 1991 : 204) investigated the chromatographic behavior of dansyl-, dinitrophenyl-, and β-naphthyl-substituted amino acids and alkaloids on layers of partially C-18 modified silica with aqueous-organic solutions containing β-cyclodextrin as chiral agent. Also, the influence of concentration of urea in the eluent was studied.

Another strategy is to use cyclodextrin as chiral information in the separation system with β-cyclodextrin bonded stationary phases. Deng and coworker (Zhu et al. 2001 : 137) prepared a phenylcarbamate substituted β-cyclodextrin bonded stationary phases and separated a large number of binaphthalene derivatives on the layer using petroleum ether-ethyl acetate-methanol mixtures as the mobile phase.

6.4 Enantiomeric separation using diastereomeric derivatives

With the increasing number of commercially available, extremely pure chiral auxiliaries, thin layer chromatographic purity control via formation of diastereomers has gained increasing importance. In contrast to direct enantiomer separations, antipode separation via diastereomers is not usually achieved with chiral adsorbents; however, enhanced “diastereomer selectivity” is also noted for asymmetrical supports.

The published work (Pflugmann et al. 1987 : 416) focuses on reactions of racemic compounds with NH_2(NH)-, OH- , and COOH- functionalities with the auxiliaries known from liquid chromatography, especially with commercial ready-to-use reagents. For example, Resolution of a racemic carboxylic acid will illustrate the specific steps in the procedure. Acids and bases are often made into diastereomeric salts.

7. Electrospinning and fiber fabrication

With all the diversity of commercial sorption materials, it is still important to perfect the available sorbents and seek new ones. In particular, Electrospinning is a process that easily produces a polymer with nanoscale fibrous structures. As a result, these fibers possess a high aspect ratio that leads to a larger specific surface. Furthermore, electrospinning has the ability to control the diameter, morphology, secondary structure, and spatial alignment of electrospun nanofibers. Although various applications have been reported recently (Jonathan and Susan 2009 : 4121), there is no report now related to the separation of the drugs using cellulose acetate fibers as stationary phase on thin layer chromatography. Therefore, nanofibers prepared by electrospinning may be good candidates for sorbents used in thin layer chromatography.

7.1 Electrospinning technique

Electrospinning has been recognized as an efficient technique for the fabrication of polymer nanofibers. Various polymers have been successfully electrospun into ultrafine fibers in recent years mostly in solvent solution and some in melt form. Potential applications based on such fibers specifically their use as reinforcement in nanocomposite development have been realized.

7.2 Equipment of electrospinning process

A typical electrospinning set-up consists of a high voltage power supply (Gamma High Voltage), a programmable syringe pump syringe, needle and a grounded collector screen. During the process a polymer solution or composite mixture is injected from a small nozzle under the influence of a voltage as high as 30 kV. The build up of electrostatic charges on the surface of a liquid droplet induces the formation of a jet. The jet is subsequently stretched to form a continuous fiber. The solvent evaporates before it reaches the collecting screen and solid fibers are collected on a conductor surface, and form nonwoven mats.

A conventional electrospinning set-up is presented in Figure 7. The advantage of the vertical system is allowing the polymer fluid to drop with help of gravity onto the collector underneath (Koombhongse et al 2001 : 3018). Some groups tilted the capillary at a defined angle to control the spinning rate (Buer et al. 2001 :

323), but introduction of the programmable syringe pump ensures a very specific controlled spinning rate can be achieved. To date there has been no systematic study comparing the fibers resulting from vertical to horizontal set-ups. The high voltage electrode can be inserted either in the polymer fluid or attached onto the tip of a metal needle.

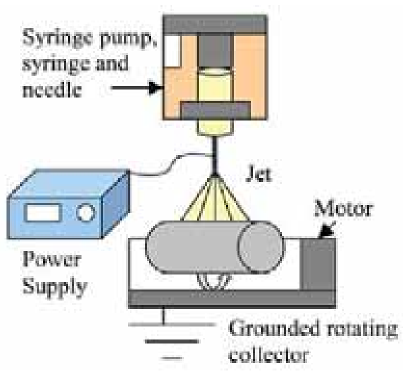


Figure 7 Electrospinning set-up: vertical and horizontal.
Source: Katarzyna M. S.and Perena G., "Electrospun composite nanofibers for functional applications," Journal of Nanoparticle Research 8 (2006) : 769.

7.3 Solution and process parameters

Theron et al. reported a systematic investigation of effect the variation of governing parameters has on the electrospinning of polyethylene oxide (PEO), polyacrylic acid (PAA), polyvinyl alcohol (PVA), polyurethane (PU), and polycaprolactone (PCL) solutions (Theron et al. 2004 : 2017). It was found the volume charge density decreases with increasing spinning for all the solutions tested. The surface charge density decreased with increasing spinning rate and voltage applied. The electric current in the jet increased with spinning rate for PEO, PVA, PAA and PU, but decreased for PCL. The volumetric spinning rate in the higher molecular weight solution of PAA had no effect on the electric current. At increased values of the volumetric spinning rate the faster solution discharge at the tip of the needle limits the solution's exposure time to the electric charge applied. The fact that the volume charge density decreased exponentially as the nozzle to ground distance increased was explained by the decrease in electric field strength.

Addition of ethanol to an aqueous PEO solution decreased the volume charge density when other parameters were kept constant, achieving higher evaporation rate, which facilitates nanofiber solidification. Deitzel et al. investigated the formation of beads and found out that the spinning voltage influences mainly the formation of beads while the polymer concentration has effect on the fiber size (Deitzel et al. : 2001 : 261)(see Table 5). Fiber diameter increased with increasing polymer concentration according to a power law relationship. At high concentrations a bimodal distribution of the fiber sizes was observed. Wilkes' group has studied the effect of solution concentration, needle–screen distance, electric potential at the tip, and spinning rate on the electrospun nanofibers (Gupta and Wilkes 2003 : 6353).

Bead structures appeared when the needle to collector distance was decreased with an increase in the average fiber diameter.Higher concentration solutions formed fibers of increased average diameter. Bead like structure was observed to turn into blobs at lower capillary–screen distance. Finally, increasing the potential decreased the fiber diameter. A lower and upper limit of polymer concentration was determined. High concentration led to failure as the viscosity was too high. Low concentration lacked enough viscosity, and resulted in a high spinning rate. The electrospinning solution must have a polymer concentration high enough to cause entanglements with viscosity low enough to allow motion induced by the electric field. To prevent the jet from collapsing into droplets before solvent evaporates the surface tension must be low. Morphological changes can occur upon decreasing the distance between needle and the substrate. The increase of needle to collector distance or a decrease in the electrical field will result in reduced bead density, regardless of the polymer solution concentration. Applied electric field can influence the morphology in periodic ways, creating a variety of new shapes on the surface (Fong et al. 1999 : 3488) (see Table 5).

Demir et al. found that the fiber diameter was proportional to the cube of the polymer concentration (Demir et al., 2002). While electrospinning polyurethane nanofibers, the authors recognized that the fiber diameters obtained from the polymersolution at a 70°C temperature were much more uniform than those obtained at room temperature. The viscosity for same concentration solutions at higher

temperature was noted to be significantly lower than that at room temperature. Increasing the electrospinning solution temperature controls the morphological imperfections such as beads or curly fibers. The increase of electrical potential resulted in rougher nanofibers (see Table 6).

Table 5 Electrospinning process parameters

Needle to collector distance	- Exponentially inverse to the volume charge density - Inversely proportional to bead formation density - Inverse to the electric field strength
Spinning rate	- Inversely proportional to fiber diameter - Directly proportional to the electric current - Directly proportional to the fiber diameter - Inversely related to volume charge density
Voltage	- Inversely proportional to surface charge density - Direct effect on bead formation - AC potential improved fiber uniformity - Inversely related to fiber diameter

Source: Katarzyna M. S.and Perena G., “Electrospun composite nanofibers for functional applications,” Journal of Nanoparticle Research 8 (2006) : 769.

Table 6 Electrospinning solution parameters

Concentration of polymer	- Directly proportional to the fiber diameter - Power law relation to the fiber diameter - Cube of polymer concentration proportional to diameter - Parabolic – upper and lower limit relation to diameter
Ionic strength	- Directly proportional to charge density - Inversely proportional to bead density
Solvent	- Effects volume charge density - Directly related to the evaporation and solidification rate
Temperature	- Inversely proportional to viscosity - Uniform fibers with less beading
Viscosity	- Parabolic relation to diameter, and spinning ability

Source: Katarzyna M. S.and Perena G., “Electrospun composite nanofibers for functional applications,” Journal of Nanoparticle Research 8 (2006) : 769.

8. Electrospinning of cellulose acetate nanofibers

As one of the most abundant renewable polymer resources, cellulose acetate (CA) has been widely used for fibers and films. Recently, the electrospinning of cellulose acetate nanofibers has attracted a great deal of attention due to their good thermal stability, chemical resistance, biodegradability, etc. These properties will ensure that they find a broad range of applications in affinity membranes, biosensors, chemosensors, protective cloths, nanocomposites, etc. Chemical structure of cellulose and their derivatives are showed in figure 8,9 and 10.

Figure 8 chemical structure of cellulose

Figure 9 chemical structure of cellulose acetate

Figure 10 chemical structure of triacetylcellulose

Cellulose acetate, first prepared by Paul Schützenberger in 1865, is the acetate ester of cellulose, the primary structural component of the cell wall of green plants and one of the most common biopolymers on earth. CA has been fabricated as semi-permeable membranes for separation processes and as fibers and films for biomedical applications. Electrospinning of 5 and 8% (w/w) CA solutions in acetone resulted in the formation of short and beaded fibers with diameters of ~1 µm, possibly because of both the low shear viscosity and the low boiling point of acetone (Jaeger et al. 1998 : 141).Improved electrospinning of CA was achieved when a binary mixture of 2:1 acetone–dimethylacetamide (DMAc) was used as the solvent (Liu and Hsieh 2002 : 2119). This mixture enabled the resulting CA solutions with concentrations in the range 12.5–20% (w/w) to be continuously spun into fibers with diameters ranging between ~100 nm and ~1 µm. CA solutions in a mixture of acetone and water with a water content in the range of 10–15% (w/w) could also be spun into ultrafine fibers (Son et al. 2004 : 5).

Furthermore, electrospinning of a CA solution in acetone and water under acidic conditions produced larger fibers whereas use of the solution under basic conditions produced much finer fibers (Son et al.2004). In addition to the different binary mixtures, a ternary mixture of 3:1:1 acetone –dimethylformamide (DMF)–trifluoroethylene (TFE) could be used to prepare a CA solution that resulted in electrospun fibers with diameters ranging from ~200 nm to ~1 µm (Ma et al. 2005 : 115).

With the objective of developing electrospun CA fiber mats as carriers for topical and/or transdermal release of plant extracts, a wider selection of solvents and mixed solvents that resulted in continuous electrospinning of CA fibers needed to be explored more extensively, because the solvents normally used for extraction of plants are hexane, dichloromethane (DCM), ethyl acetate (EA), ethanol, and methanol (MeOH) (listed in an order of increasing polarity).

Another group (Tungprapa et al. 2007 : 563) focused on the effects of solvent system, solution concentration, and electrostatic field strength(EFS) on the morphological appearance and/or size of as-spun CA products. The single-solvent systems investigated in this work were acetone, chloroform, N,N-dimethylformamide

(DMF), DCM, MeOH, formic acid, and pyridine. The mixed-solvent systems were acetone–DMAc, chloroform–MeOH, and DCM–MeOH. Particular interest was devoted to the presence in the mixed solvents of solvents with a high dielectric constant, e.g. DMAc and MeOH, that helped improve the electro-spinnability of the resulting CA solutions.

CHAPTER 3
MATERIAL AND METHODS

1. Materials

1.1 Chemicals and equipments for electrospining and polymer characterization

- Cellulose acetate (CA); white powder; $M_w \approx 30,000$ Da; acetyl content = 39.7 wt.%) (Sigma-Aldrich, co., St. Louis, MO, USA)
- N, N-dimethylacetamide (DMAc) (Sigma®, Steinheirn, Germany)
- Acetone (Merck®, Darmstadt, Germany)
- Analytical balance (Sartorius CP224S; Scientific promotion co., Ltd.)
- High voltage supply (Gamma high voltge, Ormond Beach, FL).
- Speed control motor (Oriental motor co., Ltd.)
- Scanning electron microscope (JEOL, JSM-6400,)
- Brookfield DV-III programmable viscometer (Brookfield Engineering Lab., USA)
- ECtestr11+ conductivity meter (Eutech.instrument, Malaysia)

1.2 Chemicals and equipments for chromatographic separation

- Dexamethasone (Sigma-Aldrich, co., St. Louis, MO, USA)
- Prednisolone (Sigma-Aldrich, co., St. Louis, MO, USA)
- (R)-propranolol HCl (Sigma-Aldrich, co., St. Louis, MO, USA)
- (S)-Propranolol HCl (Sigma-Aldrich, co., St. Louis, MO, USA)
- (L)-Proline (Sigma-Aldrich, co., St. Louis, MO, USA)
- (S)-Manderic acid (Sigma-Aldrich, co., St. Louis, MO, USA)
- 2-Hydroxypropyl-β-cyclodextrin (Fluka®, Buchs, Belgium.)
- Copper sulfate.5H_2O (The Government Pharmaceutical Organization, Thailand)
- Tetrazolium blue (Fluka®, Buchs, Belgium.)

- Sodium hydroxide (P. C. Drug center co., Ltd.)
- Anisaldehyde (Fluka®, Buchs, Belgium.)
- Triethylamine (Fluka®, Buchs, Belgium.)
- All mobile phase (i.e., methanol, ethanol, buthanol) (Merck®, Germany)
- Professional pH meter PP-15 (Scientific promotion co., Ltd)

2. Methods

2.1 Electrospinning of cellulose acetate fibers

2.1.1 Preparation and characterization of cellulose acetate solutions

1.7 g CA powder was dissolved in 2:1, 1:1, and 1:2 (v/v) acetone-DMAc to prepare the CA solutions at a fixed concentration of 17% (w/v) and then the CA solution was stirred for 1 h at room temperature. Before electrospinning, shear viscosity and conductivity of all the solutions were measured by using of a Brookfield DV-III programmable viscometer and a ECtestr11+ conductivity, at 25 °C, respectively.

2.1.2 Preparation of electrospun fiber plates

Electrospun fiber plates (15 x 9 cm^2) were prepared by the following procedures. Firstly, the CA solution was filled in a 5-mL glass syringe with a blunt-ended aluminium needle as a nozzle. The distance from a tip to aluminium collector was 15 cm. The feeding rate of the CA solution was varied between 0.2 and 1.0 mL h^{-1} by means of a Kd Scientific syringe pump. The CA solutions were electrospun at a positive voltage of 17.5 kV from a high voltage supply (Gamma high voltge, Ormond Beach, FL) onto aluminium plate on a drum which acted as a ground electrode. The range of studied time for electrospinning deposited on a rotating collection plate was 2 to 6 h. All of the electrospinning procedures were carried out at 25 °C. The schematic drawing of the electrospinning apparatus is illustrated in Fig. 11. Prior to use for TLC purpose, the electrospun fiber plates were immersed in n-butanol for 5 min and then dried to increase the attachment of fiber layer to the plate. Finally, electrospun fiber plates were cut into rectangular plates that were roughly 4 × 8.5 cm^2. Care was taken during the cutting of the

plate to ensure that the stationary phase was not damaged or pulled away from the aluminum plate substrate.

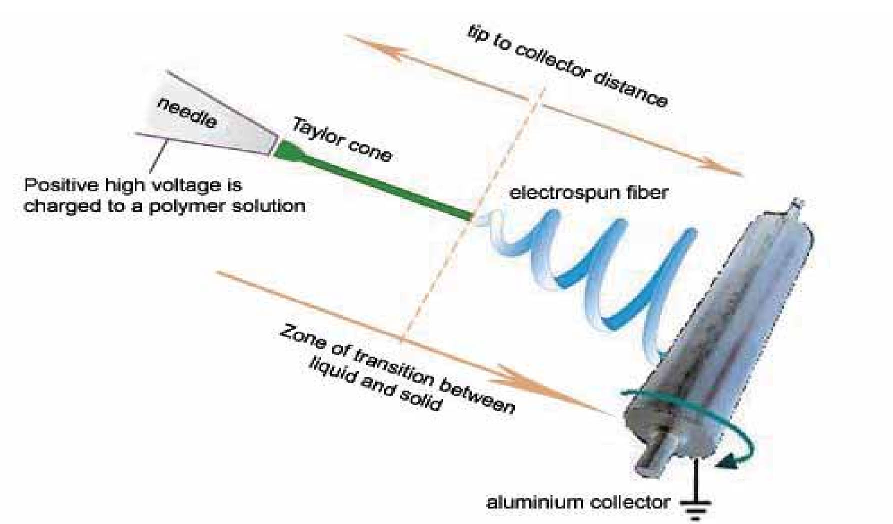


Figure 11 Schematic drawing of the electrospinning apparatus utilized in this work

2.1.3 Preparation of CA electrospun fiber plates containing chiral selectors

To prepare CA plates for enantiomeric separation, selected chiral selectors i.e. L-Amino acids, L-mandelic acid or (2-hydroxypropyl)-β-cyclodextrin were incorporated into the CA solution prior to electrospinning. This was done by dissolving 1.7 g of CA powder and chiral selectors at 1.2 % (by weight based on the weight of CA powder) in 10 mL 1:2 v/v acetone-DMAc. These mixtures were constantly stirred into clear solutions for 3 h and subjected to electrospinning under the same electrospinning parameters as mentioned above (except for spinning rate and collection time which were set at 0.4 mL h^{-1} and 6 hours, respectively.)

2.2 Preparation of CA plates by conventional spreading method

A slurry of CA was prepared by mixing 30 g CA powder which was previously ground and sieved (0.075 mm diameter) with 4.5 g calcium sulfate ($CaSO_4.1/2 H_2O$) in 60 mL distilled water and 10 mL methanol and then blended in a mortar. Air bubbles were removed by careful covering this suspension with 2-3 mL methanol and shaking. The obtained bubble-free slurry was spreaded on to a plates within 10 min. (Joseph 1992 : 31)

2.3 Characterization of electrospun fibers

2.3.1 Study of morphology

The morphological appearance and size of the electrospun fibers were determined by using a JEOL JSM-6400 scanning electron microscope (SEM). The specimens for SEM observation were prepared by cutting an aluminium plate covered with the CA electrospun fibers and then coating with a thin layer of gold using a JEOL JFC-1100E sputtering device. Diameters of the fibers and thicknesses of fiber plates were directly measured from selected SEM images using a jmicrovision software.

2.3.2 Study of solubility of electrospun fibers in various solvents

To obtain the information about the solvent resistance property of CA fibers which are required for the mobile phase selection, the solubility of electrospun fibers in various solvents was investigated. The experiment was carried out by dipping the fiber plates in different solvents i.e., hexane, diethyl ether, toluene, benzene, ethyl acetate, chloroform, tetrahydrofuran, dichloromethane, acetone, 1,4-dioxane, pyridine, dimethyl sulfoxide, dimethylformamide, acetic acid, n-butanol, isopropanol, n-propanol, ethanol, methanol, formic acid and water for 20 min. The solubility behavior was then observed and the results were compared to Hansen’s theory of solubility.

2.4 Study of chromatographic separation using CA electrospun fiber plates

2.4.1 Non-chiral separation of steroidal sample

2.4.1.1 Separation on electrospun fiber plate

Electrospun fiber plates (4 x 8.5 cm^2) were used to separate a mixture of two steroids. Aliquots (0.1 µL) of standard steroid solutions (1µg/µL dexamethasone, 1µg/µL prednisolone and mixture of 1µg/µL dexamethasone and prednisolone) were spotted 1 cm from the bottom at three locations across the plates by 2.5 µL micropipette. Binary solvent systems containing water - methanol were used and the influence of different ratio (0:100, 10:90, 20:80, 30:70, 40:60, 50:50) on resolution were investigated. Five hundred milliliter beakers were preequilibrated with the mobile phase at 20±2 $^{\circ}$C for 20–30 min prior to placing the plates inside. The separation was run until the front of mobile phase had moved 7 cm from the origin. The spot detection was done by spraying the plate with mixture solution of 0.2% w/v tetrazolium blue and 2% w/v sodium hydroxide in methanol. The chromatographic performance was evaluated by the observation of spot shape and separation as well as the running time used. The parameters including retardation factor, resolution, and efficiency were also calculated.

The basic parameter used to describe migration in TLC is the R_f value, where

$$R_f = \frac{\text{distance moved by the solute}}{\text{distance moved by mobile phase front}} \qquad (1)$$

The mathematical description of resolution is R. The Resolution of two adjacent chromatographic lower spot and upper spot is defined as being equal to the distance between the two spot centers ($Z_2 - Z_1$) divided by the mean spot widths (eq. 2)

$$R = \frac{Z_2 - Z_1}{0.5(w_1 + w_2)} \qquad (2)$$

The efficiency of a TLC separation is described by eq 1, where Z_S is the distance traveled by the solvent front and w is the width of the developed sample spot.

$$N = 16\left(\frac{Z_S}{w}\right)^2 \qquad (3)$$

This equation predicts that efficiency increases with the square of the ratio Z_S/w.

2.4.1.2 Separation on commercial silica plates

The separation performance on commercial plates (Silica gel 60 F_{254}) was used to compare with that on electrospun fiber plates. On these silica plates, the samples were chromatographed by using a mobile phase composing of water – methanol (40:60 v/v). Spot detection and chromatographic performance evaluation were done in the same way as the above procedures.

2.4.1.3 Separation on CA plates prepared by slurry spreading technique

The separation performance on CA plates prepared by slurry spreading technique was compared with that on electrospun fiber plates. On these plates, the samples were chromatographed by using a mobile phase composing of water – methanol (40:60 v/v). Spot detection and chromatographic performance evaluation were done in the same way as the above procedures.

2.4.2 Enantioseparation of chiral drugs

Solutions (2 µg/µL) of (±)-propranolol HCl were prepared in methanol and solutions of optically pure (S)-isomers and pure (R)-isomers were also prepared at the same concentration (1 µg/µL). Electrospun fiber plate containing chiral selectors (from method 2.1.3) were used to separate a racemic mixture of (±)-propranolol HCl. Solution of racemic mixture and its pure isomers were spotted (0.1 µL) side by side with the aid of 2.5 µL micropipette, 1 cm from the bottom at three locations across the plates. These were preequilibrated with the mobile phase at 20±2 ◦C for 20–30 min.

Plates were developed 7 cm from the origin with various solvent systems e.g. BuOH, 2-Propanol-Hexane (1:1), BuOH-Hexane-H_2O (8:1:1), BuOH-Triethylamine-H_2O (8:1:1) and BuOH-Acetic â-H_2O (8:1:1) which did not damage electrospun CA plate. Finally, all spots were located by spraying 5% w/v iodine solution in methanol. Evaluation was done in the same way as non-chiral separation.

CHAPTER 4
RESULTS AND DISCUSSION

1. Characterization of CA solution

Before the electrospinning, shear viscosity and conductivity of 17% (w/v) CA solutions prepared in 2:1, 1:1, and 1:2 (v/v) acetone–DMAc were investigated. The results are summarized in Table 7. It was found that both shear viscosity and conductivity of the solutions decreased with increasing acetone content. This observation was probably due to a lower shear viscosity and surface tension of acetone solvent, compared with DMAc (Table 8). Despite of different viscosity and surface tension, all solutions could be electrospun and thus used to prepare the TLC plates.

Table 7 Shear viscosity and conductivity of cellulose acetate solutions prepared in the different solvent ratios.

Ratio of acetone–DMAc	Surface tension (mN m^{-1})	Shear Viscosity (mPa.s)	Electrical conductivity (μS cm^{-1})
2:1	34.69 ± 0.09	1147.81 ± 2.87	6.1 ± 0.01
1:1	35.60 ± 0.12	1321.07 ± 0.01	6.4 ± 0.01
1:2	37.08 ± 0.17	1428.45 ± 0.94	6.6 ± 0.01

Table 8 Properties of the solvents used in this work.

Solvent	Density ($g\ cm^{-3}$)	Boiling point (^oC)	Dipole moment (Debye)	Dielectric constant	Surface tension ($mN\ m^{-1}$)	Shear viscosity (mPa.s)
Acetone	0.786	56.3	2.70	20.7	31.6	0.304
DMAc	0.937	166.0	3.72	37.8	38.4	1.020

Source: Tungprapa, S., T. Puangparn, M. Weerasombut, I. Jangchud, P. Fakum, S. Semongkhol, C. Meechaisue, and P. Supaphol. "Electrospun cellulose acetate fibers: effect of solvent system on morphology and fiber diameter." Cellulose (2007) 14 : 563–575.

2. Characterization of electrospun fibers

2.1 Effect of acetone-DMAc content in solvent on the morphology of electrospun nanofibers

Figure 12 shows SEM images of the electrospun fibers obtained from the CA solution composed of different solvent ratios but prepared under the same electric field (17.5 kV/15 cm). Obviously, different microscopic appearance consisting of fibers and beads was seen. The CA solution prepared in 2:1 (v/v) acetone-DMAc yielded uniform bead-free fibers. On the contrary, when 1:1 and 1:2 (v/v) acetone-DMAc were used as solvents at the same polymer concentration, fibers with numerous spindle or spherical beads were obtained, respectively. Generally, bead formation in electrospinning is known to cause by several parameters such as viscosity, conductivity, surface tension and evaporation of solution or solvent (Theron et al. 2004 : 2017). For instance, higher bead density is likely to occur when a solvent with low volatility is used. In our case, the evaporation rate should also be the most significant factor accountable for the fiber morphology because the boiling point of acetone (56.3 ^oC) is highly different from that of DMAc (166.0 ^oC). Therefore, CA solution with high acetone content such as 2:1 (v/v) acetone-DMAc was prone to evaporate at faste rate and eventually produced electrospun fibers without beads. Furthermore, the shape of the electrospun fibers was transformed from connected

spheres to smooth fibers can be attributed to the reduction of surface tension of the solution resulting from adding acetone. Because acetone has much lower surface tension than DMAc as mentioned earlier, the more acetone was added into DMAc, the lower surface tension of the mixture was achieved.

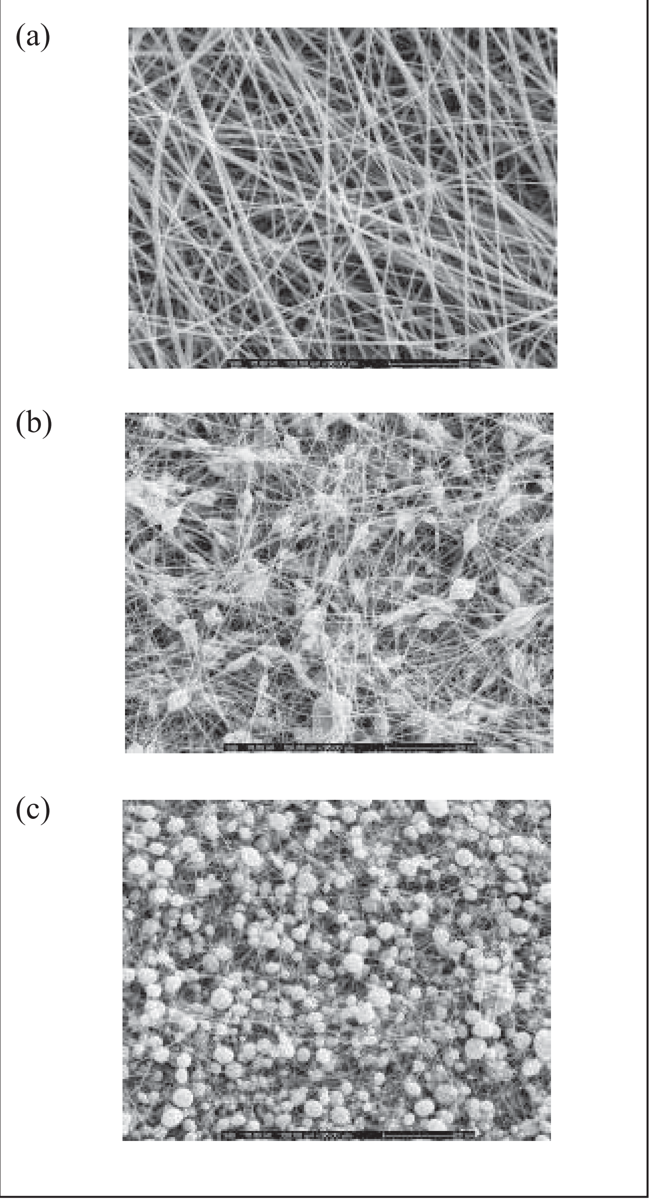


Figure 12 SEM images of 17% CA (w/v) nanofibers spun from the solution containing different compositions of the mixed solvents; (a) acetone-DMAc (2:1 v/v), (b) acetone-DMAc (1:1 v/v), (c) acetone-DMA (1:2 v/v).

2.2 Effect of spinning rate on the morphology of electrospun nanofiber

SEM images of electrospun CA fiber mats obtained from the spinning different rates are shown in Table 9 and the diameters of fibers and beads are summarized in Table 10. The experimental results revealed that diameter of fibers obtained from 17% CA solution in 2:1(v/v) acetone–DMAc increased with an increasing spinning rate (Table 10). In cases of beaded fibers which were obtained from CA dissolved in 1:1 and 1:2 (v/v) acetone–DMAc, the enlargement of beads were also observed. This finding was in agreement with Sawicka et al. who reported that spinning rate was directly proportional to the fiber diameter because high spinning rate would lower the volume charge density (Sawicka et al. 2005 : 585). By using a faster spinning, the rate of evaporation decreased and the volume of the solution would be high. As a result, the volume charge density decreases and the bending speed will be smaller, leading to larger fiber diameter.

Table 9 Selected SEM images of electrospun CA fiber mats at various spinning rates and various ratio of the mixed solvents

Ratios of acetone-DMAc solvent	Spinning rates (ml h^{-1})		
	0.2	0.6	1.0
2:1	20μm	20μm	20μm
1:1	20μm	20μm	20μm
1:2	20μm	20μm	20μm

Note: 17% (w/v) CA solution was applied at electric field = 17.5 kV/15 cm and the spinning time = 6 h.

Table 10 Diameters of the individual fibers and beads within the resulting electrospun fiber mats (n = 100)

Ratios of acetone-DMAc solvent(v/v)	spinning rate (ml h^{-1})	Fiber diameters (μm)	Bead diameters (μm)
2:1	0.2	0.40 ± 0.15	-
	0.4	0.56 ± 0.22	-
	0.6	0.59 ± 0.24	-
	0.8	0.65 ± 0.31	-
	1.0	0.66 ± 0.41	-
1:1	0.2	0.17 ± 0.05	1.11 ± 0.42
	0.4	0.17 ± 0.04	1.60 ± 0.81
	0.6	0.17 ± 0.04	2.35 ± 1.08
	0.8	0.17 ± 0.06	2.39 ± 1.36
	1.0	0.17 ± 0.05	3.40 ± 1.74
1:2	0.2	0.13 ± 0.03	0.98 ± 0.43
	0.4	0.12 ± 0.03	1.41 ± 0.87
	0.6	0.11 ± 0.02	1.77 ± 1.08
	0.8	0.11 ± 0.03	2.64 ± 1.14
	1.0	0.12 ± 0.02	3.73 ± 1.67

Note: 17% (w/v) CA solution was applied at electric field = 17.5 kV/15 cm

2.3 Effect of spinning time and spinning rate on the thickness of electrospun fiber mat

Once the electrospinning had finished, the fiber layers collected on most areas of the aluminium plates were found to have similar thickness by visual observation, except for those located near the plate border which were often thinner. Thus, for the further use as efficient TLC device and for the measurement of fiber mats' thickness, the fiber plates were cut by selecting the areas in which the mats had even thickness. The results of the thickness measurements and side-viewed illustrations are shown in Table 11 and 12. It was found that thickness were directly proportional to the spinning time and rate. Interestingly, too longer spinning time (e.g. over 12 h) sometimes caused the detachment of the fiber mat from the backing (data not shown). This problem was more pronounced in the case of bead-free fibers than bead containing fibers.

Table 11 The SEM image and results of the thickness measurements (n = 100) at 5 various spinning time

Ratios of acetone-DMAc solvent	Spinning time (hour)		
	2	4	6
2:1	Thickness 18.84 ± 0.53	Thickness21.39 ± 0.61	Thickness 46.13 ± 0.84
1:1	Thickness 10.79 ± 0.56	Thickness 15.58 ± 0.67	Thickness 23.23 ± 0.76
1:2	Thickness 8.03 ± 0.75	Thickness 9.35 ± 0.73	Thickness14.93 ± 0.88

Note: 17% (w/v) CA solution was applied at electric field = 17.5 kV/15 cm and the spinning rate = 0.6 mL h^{-1}.

Table 12 The SEM image and results of the thickness measurements (n = 100) at various spinning rates

Ratios of acetone-DMAc solvent	Spinning rates (mL h^{-1})		
	0.2	0.6	1.0
2:1	Thickness 34.00 ± 0.85	Thickness 46.13 ± 0.84	Thickness 74.88 ± 1.23
1:1	Thickness11.26 ± 0.89	Thickness 23.23 ± 0.76	Thickness 37.54 ± 0.99
1:2	Thickness 7.58 ± 0.60	Thickness 14.93 ± 0.88	Thickness 26.31 ± 1.78

Note: 17% (w/v) CA solution was applied at electric field = 17.5 kV/15 cm and the spinning time = 6 h.

2.4 Study of solubility of electrospun fiber mats in mobile phase

Appropriate mobile phase compositions should not be able to dissolve cellulose acetate so mobile phase compositions were selected from solubility experiments.

As can be seen from Table 13, most of polar aprotic solvents and certain solvents e.g. acetic acid, acetonitrile, ethylacetate dissolve CA electrospun. Running with these pure solvent as mobile phase should be avoided or at least.

Table 13 Dissolution of cellulose acetate fiber mats in various solvents

Solvent	Dissolution of cellulose acetate fiber mats
Non-polar Solvents	
Hexane	Insoluble
Diethyl ether	Insoluble
Toluene	Insoluble
Benzene	Insoluble
Ethyl acetate	Soluble
Chloroform	Insoluble
Polar aprotic Solvents	
Tetrahydrofuran (THF)	Soluble
Dichloromethane (DCM)	Soluble
Acetone	Soluble
1,4-Dioxane	Insoluble
Pyridine	Soluble
Dimethyl sulfoxide (DMSO)	Soluble
Dimethylformamide (DMF)	soluble
Polar protic Solvents	
Acetic acid	Soluble
n-Butanol	Insoluble
Isopropanol	Insoluble
n-Propanol	Insoluble
Ethanol	Insoluble
Methanol	Insoluble
Formic acid	Insoluble
Water	Insoluble

3. Application of electrospun fiber plates for non-chiral chromatographic separation

3.1 General aspect of electrospun fiber plate as TLC device

In general consideration, the resulting electrospun plates were mechanically stable and could be handled in the same manner as commercially available TLC plates. In addition, the electrospun plates obtained from the CA solutions prepared by different of acetone DMAc ratio (Fig 13a, 13b, 13c) were apparently as smooth as commercial plate (Fig. 13d). In contrast, CA layers coated on the plates prepared by conventional slurry spreading technique, even with the aid of calcium sulfate binder, were uneven and easily cracked. Since butanol dipping was done after electrospinning as previously described, the morphology of the CA electrospun fiber mats before versus after this process was studied and the SEM results are shown in Table 14. Overall, no significant difference between the fiber mats before and after dipping step was observed.

In another view of point, electrospun fiber plate might have an advantage over commercial plates in that the latter often contain binders (e.g. gypsum) that often times the presence of this binder limits the chromatographic performance of the plates. The CA electrospun fiber plates can be fabricated without the use of binder and thus are not limited by the chromatographic performance of a given binder.

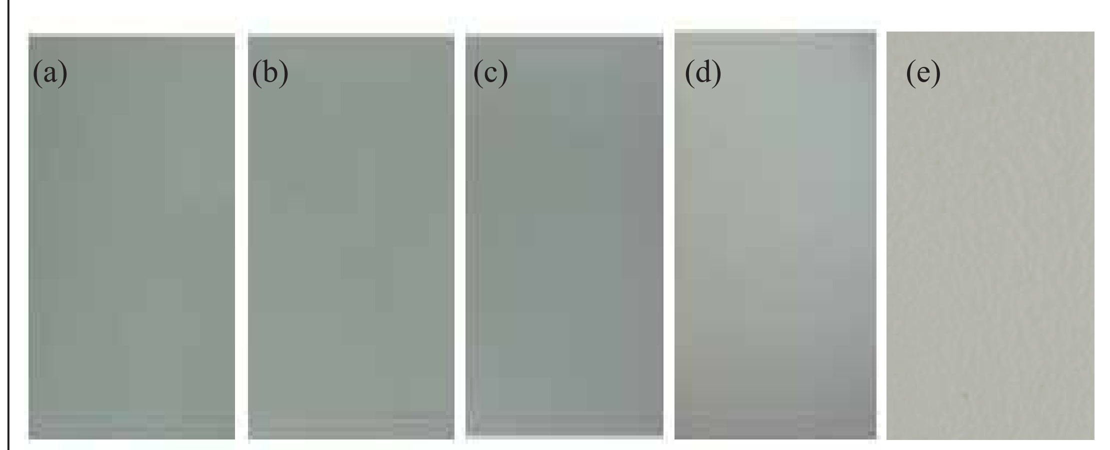


Figure 13 Appearance of plates including (a) electrospun plate from 2:1 ratio, (b) electrospun plate from 1:1 ratio, (c) electrospun plate from 1:2 ratio (d) silica commercial plate and (e) plate prepared by CA slurry spreading

Table 14 SEM micrographs of electrospun fiber mats show three morphology

Solvent of polymer solution	Acetone-DMAc 2:1(v/v)	Acetone-DMAc 1:1(v/v)	Acetone-DMAc 1:2(v/v)
Before dipping butanol			
After dipping butanol			

3.2 Selection of mobile phase

Before the CA electrospun plates were used for TLC separation of the samples, the migration behavior of solvent on the plates with different fiber morphology was examined and shown in Table 15. Surprisingly, the solvent moved evenly as a straight line on the electrospun fiber plates containing spindle beads and spherical beads along the fibers. Conversely, the solvent front appearing on the electrospun plate of smooth, bead-free fibers was irregular. This irregular movement can cause varied and non-reproducible retardation factors and resolution values if the plates are used.

Table 15 Behaviors of solvent front of the mobile phase (water-methanol 40:60) during development on different morphology of electrospun plates that were prepared under the same condition :17 % CA (w/v) solution which was applied at electric field of 17.5 kV/15 cm, spinning rate of 0.2 ml h^{-1} and spinning time of 6 hours.

Solvent of polymer solution	Acetone-DMAc 2:1(v/v)	Acetone-DMAc 1:1(v/v)	Acetone-DMAc 1:2(v/v)
Morphology	Smooth fibers	Spindle beads along the fibers	Spherical beads along the fibers
Image of solvent front			

In order to carry out the evaluation of the chromatographic performance of electrospun fiber plates, a mobile phase system was investigated as a prerequisite so that it could be used as a standard mobile phase for the subsequent studies. Hence, the aim this experiment was to find out at least one usable system. The full optimization in order to obtain the best mobile was not goal. To conduct the study, the fiber plates which were electrospun from 17% (w/v) CA in 1:2 (v/v) acetone-DMAc under the electric field = 17.5 kV/15 cm, spinning rate = 0.6 ml $h^{-1}$and spinning time = 4 h were chosen as a representative for all kinds of plates owing to their satisfactory common features i.e. they could be prepare in a short time. The separation was done on a mixture of steroids (~1μg) consisting of dexamethasone and prednisolone. Assumed that CA fibers separate the solutes via the reversed phase mechanism because the CA used contained ~40% acetyl content, aqueous solution containing moderately polar solvents should be the solvent of choice. Since water and methanol seem to be the most common solvents and are comparable with (do not dissolve) CA fibers, they were chosen for the development of binary mobile phase system. The influent of water to methanol ratio (0 – 50 %) in the mobile phase on the separation of the two steroid was investigated. It was found that to achieve the highest resolution on the selected electrospun fiber plates, the mobile phase should comprise of 40:60 water-methanol (Table 16. Fig. 16a). However, this mobile phase failed to resolve the steroids if the commercial silica plate was used (Fig. 16b). For this kind of plate, other solvent systems such as ethyl acetate-cyclohexane (75:25) are more suitable and should be used instead (Fig. 16c). The failure in the separation was also observed when this mobile phase was used with the CA plate which prepared by spreading the CA slurry since the bands of solutes disappointedly appeared as excessive tailing (16d).

As seen from the position of solute spots on the plates, it clearly confirmed that the separation on the CA electrospun fiber plate was of reversed phase mode since the migration order of the two steroids on this plate was reverse to that on the silica plate which is normal phase. On the reversed phase mode, prednisolone (Fig. 14), which is more polar than dexamethasone (Fig. 15), therefore moves faster on the plate. Since solvents with higher polarity are normally employed for the

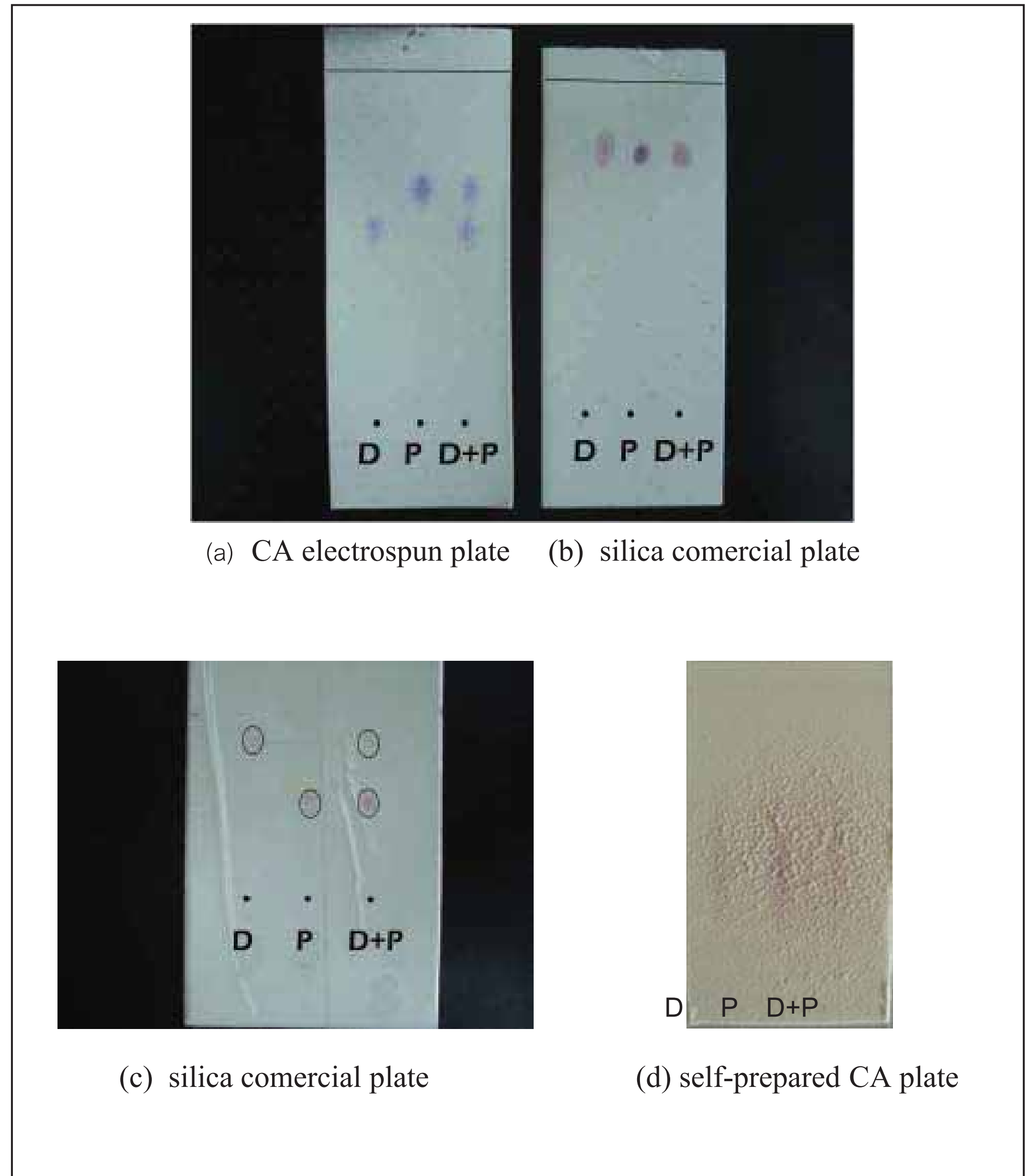
reversed phase chromatography, the proof of reverse phase nature of CA fiber plate supports our decision in choosing methanol-water as a mobile phase.

Table 16 The retention behavior of steroids chromatographed on electrospun fiber plate using mobile phase containing water to methanol in various ratio.

Water to methanol ratio	R_f of Standard compounds		Resolution
	Prednisolone	Dexamethasone	
0:100	1.000	1.000	0.00
10:90	0.826	0.867	0.429
20:80	0.586	0.640	0.714
30:70	0.547	0.667	1.18
40:60	0.545	0.709	1.33
50:50	0.240	0.456	1.00

Figure 14 Chemical structure of prednisolone

Figure 15 Chemical structure of dexamethasone



(a) CA electrospun plate (b) silica comercial plate

(c) silica comercial plate (d) self-prepared CA plate

Figure 16 Resolution of two steroids (~1μg) by electrospun fiber plate (a) in comparison with commercial silica plate (b) using water-methanol (40:60) mobile phase. Resolution of steroids on the commercial silica plate in ethyl acetate-cyclohexane (75:25) (c). Excessive tailing on self-prepared CA plates (d). note : (D = Dexamethasone, P = Prednisolone) developing distance : 7cm

3.3 Developing time on three morphology of electrospun fiber plate

The operation speed is one of the properties of the separation methods that should be concerned and any may be the limitation for the practical use of the method. For instance, cellulose triacetate TLC plate has been reported and used in some researches, but has not developed commercially until now probably due to the long developing times (time that the solvent used to migrate from the start to the end point on the plate) that are required (about 2.5 hours) for sufficient migration of analysts (Lepri 1994 : 376–381). This study was aimed to compare the speed of separation as measured by the developing time when the samples were run on electrospun fiber plates with different fiber morphology and thickness of mat. The mobile phase consisting of 40:60 water-methanol was used based on the previous experimental results which revealed that it could resolve the steroid sample effectively.

The result showed that the developing time varied depending on the morphology of fibers. In most cases where the spinning rates and times were fixed, the order od developing time that the mobile phase had used in order to move 7 cm far from the start point was as followed; fibers prepared from 2:1 acetone-DMAc > fibers prepared from 1:1 acetone-DMAc > fibers prepared from 1:2 acetone-DMAc (Table 17 and Fig. 17). In another words, the solvent migrated faster on the plate containing beaded fibers, in a comparison with bead-free fibers. It is possible that the solvent migration rate was influenced by the fibrous structure and bead structure. While small size (with high surface area) fibers and highly overlapping structure promotes the migration of solvent by means of capillary action, beads impede it. Since the bead-free fibers (obtained from 2:1 acetone-DMAc solution) had the largest diameter size (Table 12) and formed the loosest layer, they did not support an efficient capillary action, leading to the longest developing time of more than 30 min. This was different from beaded fibers (obtained from 1:1 and 2:1 acetone-DMAc solution) which have small size and more dense structure. Since the fibers obtained from 1:1 acetone-DMAc solution were slightly larger than those from 1:2 acetone-DMAc and formed a dense network inside the layer, however, with lower bead density, they compromised the presence of fibers and beads in the structure. As a result, the shortest developing time was required when the separation was done on this stationary phase. In

conclusion, the bead-free fiber mat are likely to be the least suitable TLC plate if only run time is considered.

3.4 Effect of spinning rate and time on the developing time

Among the fibers prepared from the solvent containing the same acetone-DMAc ratio, the effect of the spinning rate and time in the fiber fabrication process on the developing time in the chromatographic run was also examined. It was found that the increase of spinning rate and time which directly enhanced the thickness of resulting fiber layers shortened the developing time (Fig. 18-20). It is possible that a thicker layers of stationary phase may better act as a chromatographic bed to pull up the solvent along the plate by the capillary force.

Table 17 Developing time on electrospun fiber plate which produced at various parameters of spinning process include ratio of acetone-DMAc solvent, spinning rate and spinning time.

Acetone-DMA ratio	Developing time(minute) (TLC distance : 7cm)														
	spinning rate (mL h^{-1})														
	0.2			**0.4**			**0.6**			**0.8**			**1**		
	Spinning time (h)			Spinning time (h)			Spinning time (h)			Spinning time (h)			Spinning time (hr)		
	2	**4**	**6**	**2**	**4**	**6**	**2**	**4**	**6**	**2**	**4**	**6**	**2**	**4**	**6**
(2:1)	80	70	64	70	52	47	50	46	40	42	42	36	40	30	33
(1:1)	51	49	46	33	32	32	25	23	21	26	25	20	19	17	15
(1:2)	53	52	49	44	48	42	45	43	34	27	26	23	26	22	15

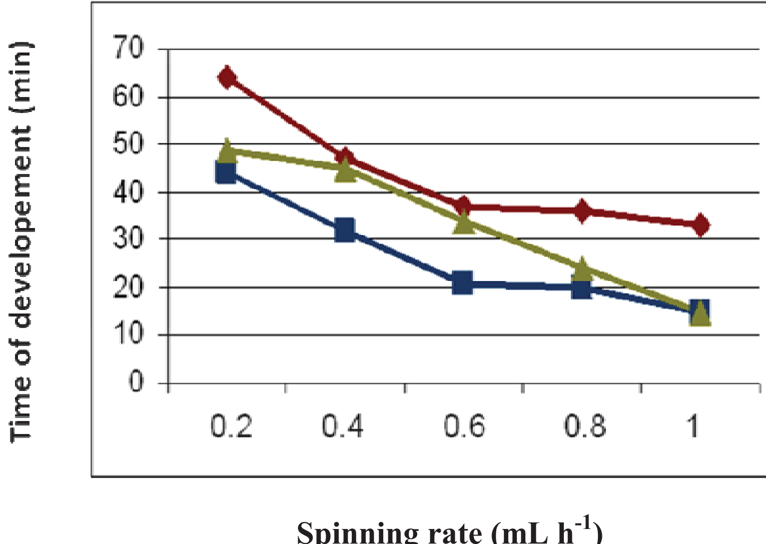


Figure 17 Dependence of the Developing times of CA electrospun fiber plates on different spinning rate of electrospinning procedure. Conditions: Stationary phase: electrospun fiber mats at spinning time of 6 hours, separation distance: 7 cm, mobile phase; water-methanol (40:60)

Ratio of acetone-DMAc (v/v) : ▲ = 2:1, ◆ = 1:2, ■ = 1:1

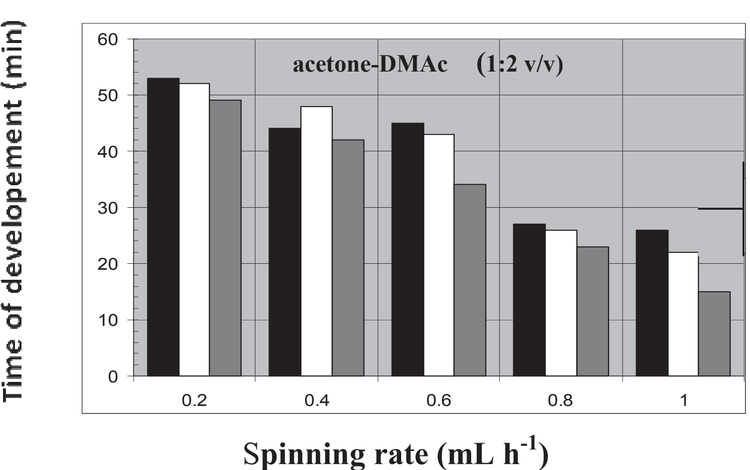


Figure 18 Dependence of the developing times on various spinning rates and Spinning times, Plate: electrospun mat from 17% w/v cellulose acetate in acetone-DMAc 1:2 v/v, spinning time : ■ = 2 h, □ = 4 h, ■ = 6 h

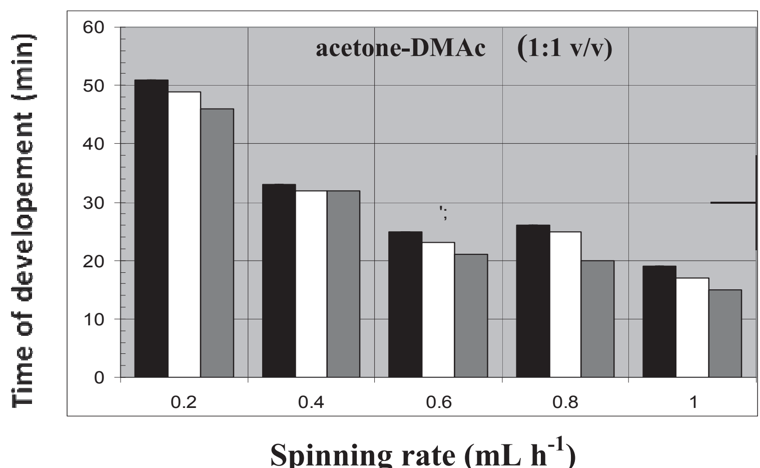


Figure 19 Dependence of the developing times on various spinning rates and spinning times, Plate: elerctrospun mat from 17% w/v cellulose acetate in acetone-DMAc 1:1 v/v, Spinning time: ■ = 2 h, □= 4 h, ■ = 6 h

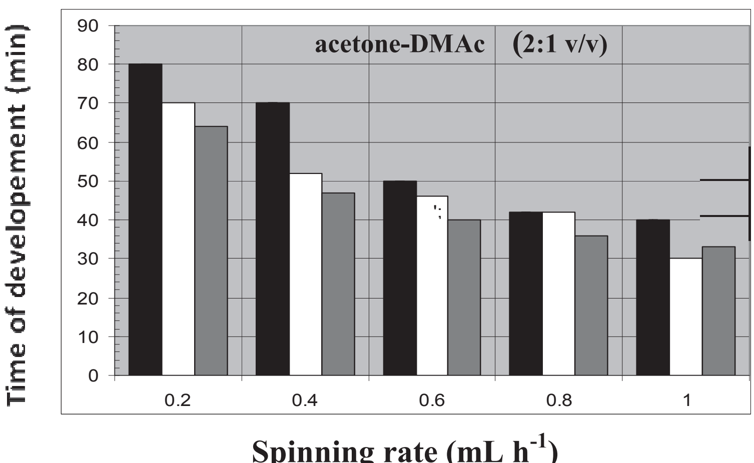


Figure 20 Dependence of the developing times on various spinning rates and spinning times, Plate: elerctrospun mat from 17% w/v cellulose acetate in acetone-DMAc 2:1 v/v, spinning time: ■ = 2 h, □= 4 h, ■ = 6 h

3.5 Chromatographic performance of CA electrospun fiber plates in the separation of steroids

Various CA electrospun fiber plates fabricated by using different parameters including the ratio of solvents (2:1, 1:1, 1:2 acetone-DMAc), spinning rate (0.2, 0.4, 0.6, 0.8, 1.0 ml h^{-1}) and spinning time (2, 4, 6 h) were tested for their performance in the separation of a mixture consisting of dexamethasone and prednisolone. The calculated chromatographic parameter i.e. R_f values, plate efficiency (N) and resolution (R) as well as the tailing trait of spots are summarized in Table 18. Clearly, it was found that fiber mats that were spun at too low spinning rate for too short time could not resolve the mixture as a result of their too thin layer. This problem was very obvious in the polymer solution formula in which 1:2 acetone-DMAc was employed because it usually yielded thinner mats than the other formulas. In another way, fiber mats produced by using too high spinning rate also failed to separate the steroids due to the tailing movement of spots. As previously shown and discussed, the high spinning rate often produced larger and more irregular size of fibers and beads. This characteristic of the stationary phase particles is known to result in an inefficient resolution.

The successful separation was obtained when the optimal range of spinning rate and time were used. These condition were slightly different depending on the formulas used for the preparation of polymer solution. For example, when 2:1 acetone-DMAc was used as a solvent, the fibers obtained from the electrospinning at 0.2 - 0.4 mL h^{-1} for 4 – 6 h worked well for the separation. However, it should be reminded that the used of bead-free fiber mats prepared from 2:1 acetone-DMAc solvent often caused the problems of unsatisfactorily long run time and uneven migration of solvent.

For the plates which were made by using the same spinning rate and capable of resolving the samples, the increased spinning time enhanced to the plate efficiency as well as the resolution. For instance, in the case of 1:1 acetone-DMAc solvent with spinning rate of 0.6 ml h^{-1}, the efficiency raised from 1,616 to 2,178 when the spinning time increased from 2 to 6 h. Also, the resolution between spots was higher (from 1.64 to 2.00 and to 2.19). This could be explained that the longer

spinning time at an appropriate spinning rate produced thicker layer of stationary phase and act better as a chromatographic bed.

To sum up the results, it was found that the 17% CA electrospun fibers prepared in solvent of optimal acetone-DMAc ratio and subsequently subjected to electrospinning at the separation of dexamethasone and prednisolone by using water-methanol mobile phase. One of the examples is the fibers prepared frome 17 % CA in 1:1 acetone-DMAc solvent with a spinning rate of 0.6 ml h^{-1} and spinning time of 4 h because they had efficiency and resolution and produced even migration of solvent in the run. In addition, the fabrication could be finished within 4 h and the separation could be achieved within about 20 min.

The results show that three morphology of electrospun stationary phase which produced under the same condition (such as 17% (w/v) CA solution was applied at electric field 17.5 kV/15 cm, spinning rate 0.4 mL h^{-1}, spinning time 6 h) are feasible for thin layer chromatography (Fig 21). As mentioned before, the solvent fronts of electrospun plates from CA solution in acetone-DMAc (2:1) cannot be assumed to be linear because irregular movement was obtained with electrospun plate of Smooth fibers (Fig 21a).

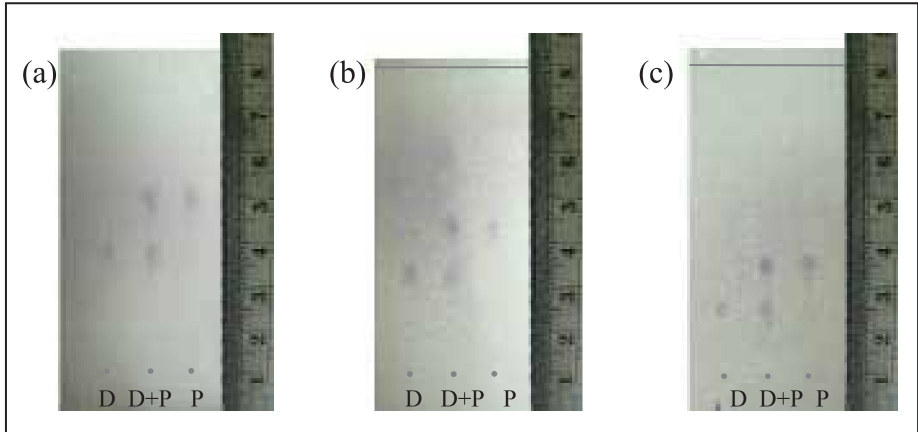


Figure 21 Figure a, b and c are electrospun plates which were prepared from CA solution in acetone-DMAc (2:1), (1:1), (1:2), repectively. All plates are dried after separation of dexamethasone (D) and prednisolone (P) with the mobile phase water–methanol (40:60).Finally, the steroids were detected with the tetrazolium blue reagent.

Table 18 Summary of the average retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in three replicate trials

Electrospun TLC plate			TLC parameter				
Ratios of acetone-DMAc solvent (v/v)	Spinning rate (ml h^{-1})	Spinning time (h)	Retardation (R_f)		Efficiency (N)		Resolution (R)
			D	P	D	P	
2:1	0.2	2	x	x	-	-	-
		4	0.45	0.59	1600	2178	1.49
		6	0.44	0.58	1685	2592	1.59
	0.4	2	x	x	-	-	-
		4	0.46	0.60	1664	2095	1.51
		6	0.44	0.59	1685	2178	1.56
	0.6	2	tailing	tailing	-	-	-
		4	tailing	tailing	-	-	-
		6	tailing	tailing	-	-	-
	0.8	2	tailing	tailing	-	-	-
		4	tailing	tailing	-	-	-
		6	tailing	tailing	-	-	-
	1.0	2	tailing	tailing	-	-	-
		4	tailing	tailing	-	-	-
		6	tailing	tailing	-	-	-
1:1	0.2	2	x	x	-	-	-
		4	x	x	-	-	-
		6	x	x	-	-	-
	0.4	2	x	x	-	-	-
		4	0.34	0.50	2592	1963	1.89
		6	0.33	0.48	2955	2316	1.97
	0.6	2	0.41	0.60	1007	1616	1.64
		4	0.38	0.56	1793	2178	2.00
		6	0.37	0.56	2178	2178	2.19
	0.8	2	tailing	tailing	-	-	-
		4	tailing	tailing	-	-	-
		6	tailing	tailing	-	-	-
	1.0	2	tailing	tailing	-	-	-
		4	tailing	tailing	-	-	-
		6	tailing	tailing	-	-	-

Table 18 (continuted)

TLC device			TLC parameter				
Ratios of acetone-DMAc solvent (v/v)	Spinning rate ($ml\ h^{-1}$)	Spinning time (h)	Retardation (R_f)		Efficiency (N)		Resolution (R)
			D	P	D	P	
1:2	0.2	2	x	x	-	-	-
		4	x	x	-	-	-
		6	x	x	-	-	-
	0.4	2	x	x	-	-	-
		4	x	x	-	-	-
		6	0.31	0.46	2592	2316	1.77
	0.6	2	x	x	-	-	-
		4	0.32	0.46	2208	2592	1.65
		6	0.20	0.33	2316	3136	1.66
	0.8	2	tailing	tailing	-	-	-
		4	tailing	tailing	-	-	-
		6	tailing	tailing	-	-	-
	1.0	2	tailing	tailing	-	-	-
		4	tailing	tailing	-	-	-
		6	tailing	tailing	-	-	-

Note: 1. (D = dexamethasone, P = prednisolone, x = The layer cannot be used for separation.)
2. 17% (w/v) CA solution was applied at electric field = 17.5 kV/15 cm

3.4 Application of electrospun fiber plates for enantioseparation

In general, three approaches have been applied to the TLC resolution of enantiomers: use of a chiral selector as impregnating reagent mixed with the adsorbent, e.g., silica gel; immersing or developing the plate in a solution of chiral selector prior to sample application; and use of a chiral mobile phase. (Yuasa et al. 1980 : 311) In this study, propranolol HCl enantiomers were resolved by using

loading/impregnating the Cu(II) complex of L-proline, L-mandelic acid and (2-hydroxypropyl)-β-cyclodextrin on CA electrospun plates via electrospinning process.

Some chromatograms of separation experiment are presented in Fig. 22, 23, 24. The results indicated that CA electrospun plate with these impregnating reagent could not separate the enantiomers of propranolol HCl due to a limited range of mobile phase tolerable by the plates. For example, aprotic solvents such as acetonitile, ethyl acetate, dichloromethane that were used for enantioseparation of propranolol in several literatures caused CA electrospun to damage.

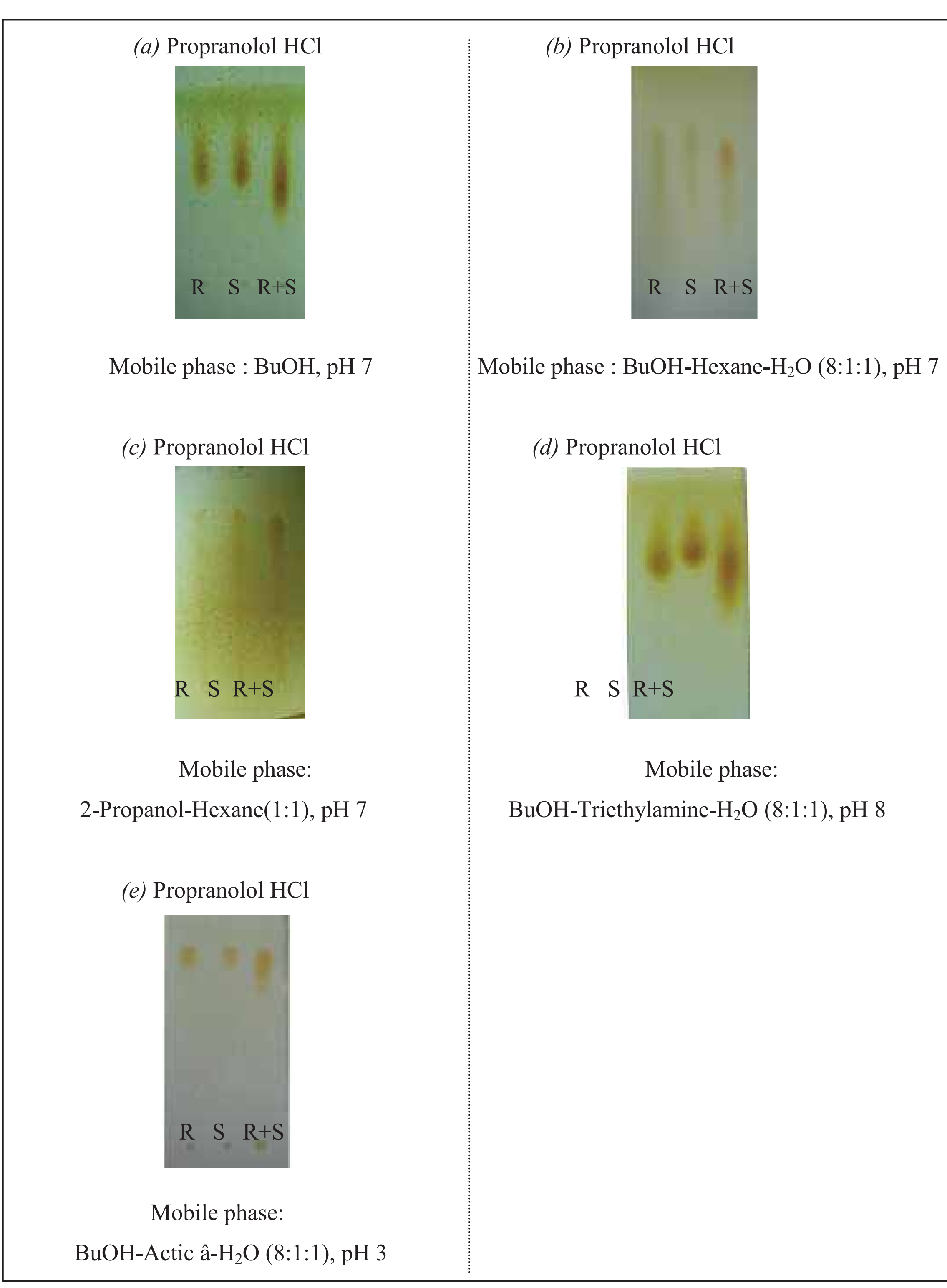


Figure 22 Photograph of the chromatogram racemic propranolol using the electrospun plate impregnated with Cu(II) complex of L-proline. The same condition including developed distance : 4 cm, temperature: 22°C, detection: iodine solution (R = R-isomer, S = S-isomer)

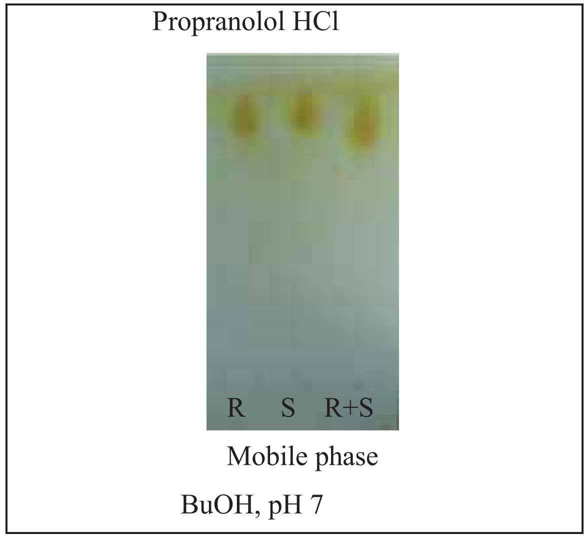


Figure 23 Photograph of the chromatogram racemic propranolol using the electrospun plate impregnated with L-manderic. The same condition including developed distance : 4 cm, temperature: 22°C, detection: iodine solution (R = R-isomer, S = S-isomer)

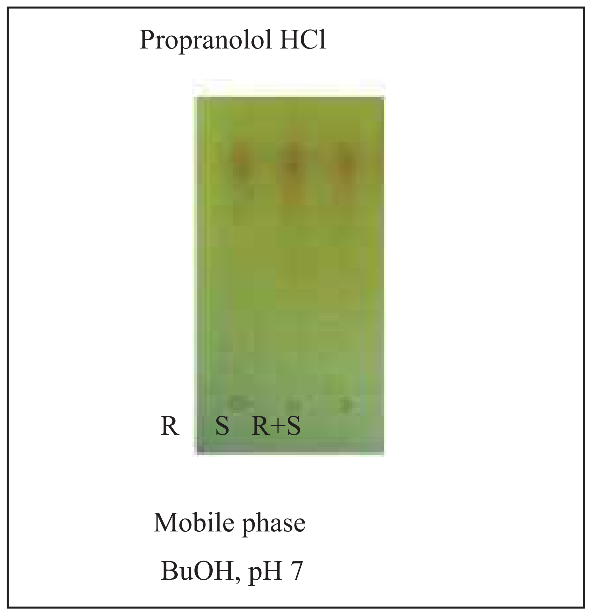


Figure 24 Photograph of the chromatogram racemic propranolol using the electrospun plate impregnated with (2-hydroxypropyl)-β-cyclodextrin. The same condition including developed distance : 4 cm, temperature: 22°C, detection: iodine solution (R = R-isomer, S = S-isomer)

CHAPTER 5
CONCLUSION

In this study, an electrospinning technique was applied for the fabrication of fiber mats aimed for the use as TLC stationary phase. Depending on the acetone-DMAc content in the CA polymer solutions, electrospun fibers with different morphologies were obtained. The acetone-DMAc ratio of 2:1 (v/v) produced smooth fibers while spindle beads along the fibers and spherical beads along the fibers were obtained when the ratio of acetone-DMAc were 1:1 (v/v) and 1:2 (v/v), repectively. The fiber layers were found to be mechanically stable and well attached to the aluminum plates, which meant that no bonding agent was required. The experimental results showed that electrospun CA mats could be applied as the stationary phase of TLC separation of dexamethasone and prednisolone, however irregular migration of mobile phase may be found on the electrospun plates with smooth fibers under some separation conditions. In term of developing time, plates with beads along the fibers usually gave shorter time than plates covered with smooth fibers. Furthermore, increasing spinning rate of electrospinning procedure shortened the developing time under the same separation conditions. Electrospun CA plates produced at high spinning rate of 0.8-1.0 ml h^{-1} caused significant tailing of the spots. Nevertheless, it was found that the electrospun fiber plates which were fabricated and subjected to the separation under suitable conditions gave satisfactory separation performance.

In addition, CA electrospun plates were investigated for chiral resolution capability for enantiomeric propranolol.HCl and cetirizine.2HCl by incorporating chiral selectors into the polymer solution prior to electrospinning. However, they did not give successful separation. Therefore, other polymers and/or mobile phase systems should be further studied for the enantioseparation purpose.

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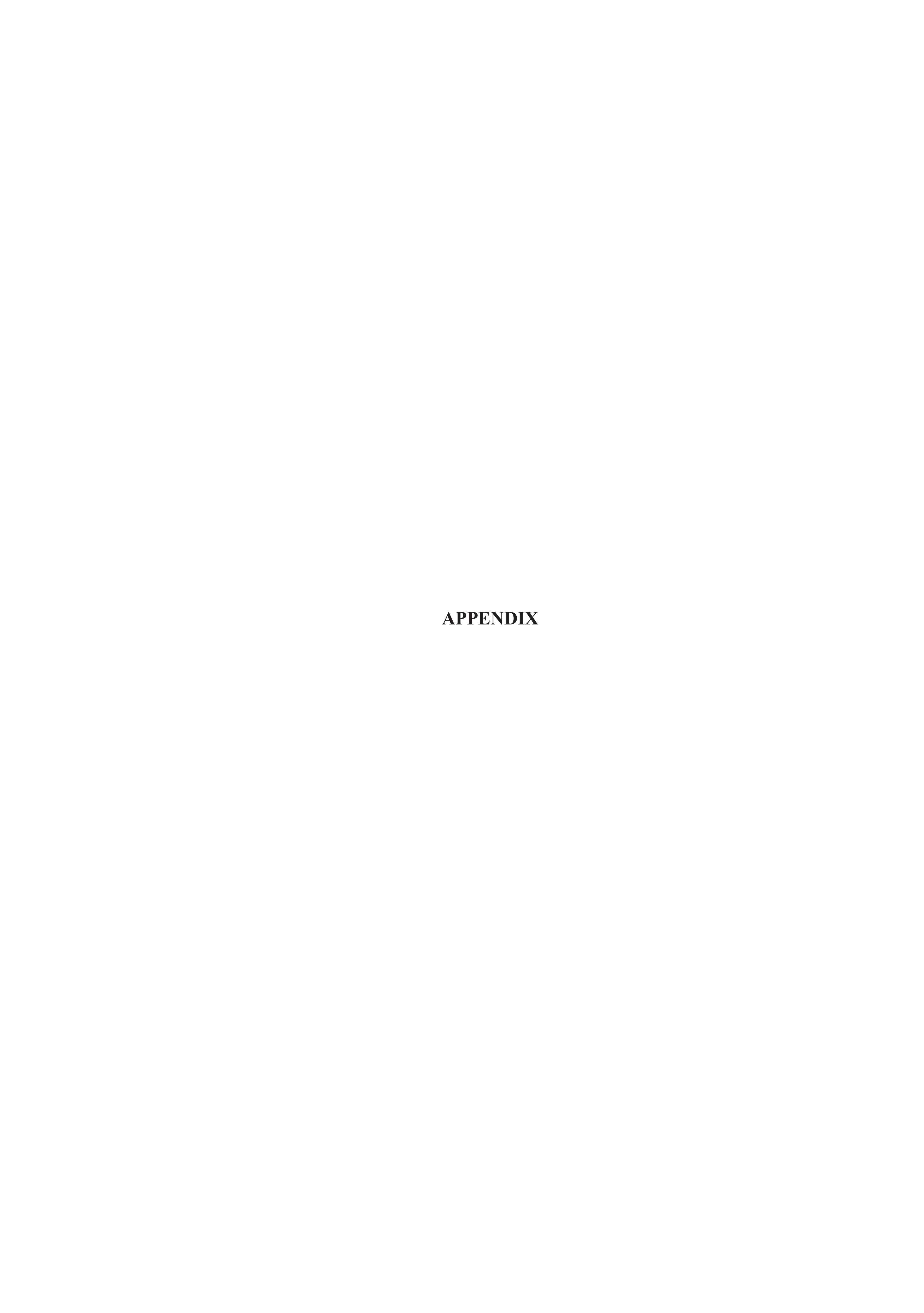
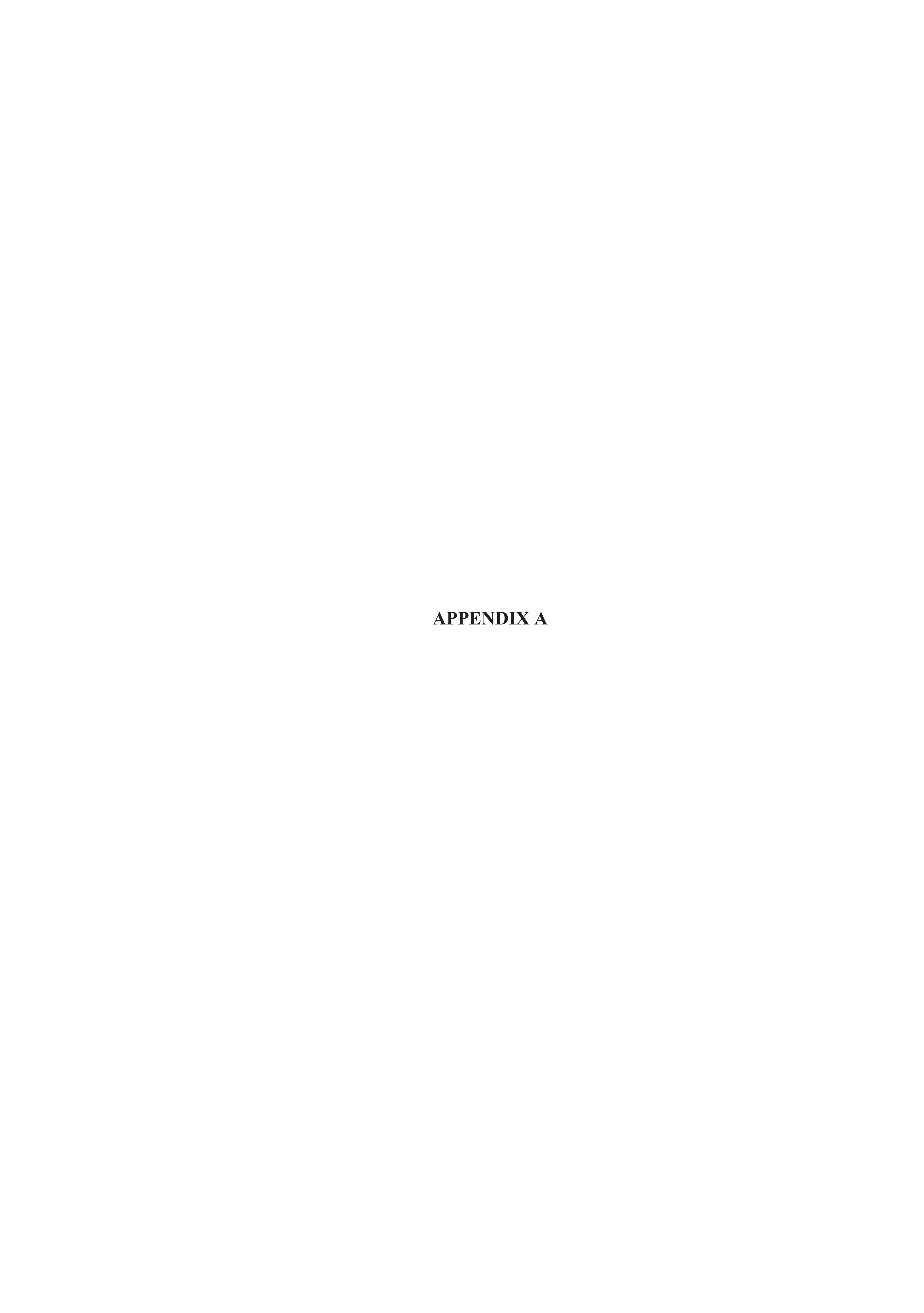


APPENDIX



APPENDIX A

Table 19 Shear viscosity of cellulose acetate solutions prepared in the different solvent ratios.

Acetone : DMAc Ratios	Conductivity (μS cm^{-1})			Average	SD
	1	2	3		
(1:2)	6.6	6.7	6.5	6.6	0.1
(1:1)	6.4	6.4	6.4	6.4	0.0
(2:1)	6.1	6.1	6.1	6.1	0.0

Table 20 conductivity of cellulose acetate solutions prepared in the different solvent ratios.

Acetone : DMAc Ratios	surface tention (mNm^{-1})			Average	SD
	1	2	3		
Acetone	31.26	31.47	31.50	31.41	0.13
DMAc	38.12	38.63	38.52	38.42	0.27
(1:2)	36.91	37.08	37.24	37.08	0.17
(1:1)	35.71	35.61	35.48	35.60	0.12
(2:1)	34.64	34.63	34.79	34.69	0.09

Table 21 Surface tention of cellulose acetate solutions prepared in the different solvent ratios.

Acetone : DMAc Ratios	surface tention (mNm^{-1})			Average	SD
	1	2	3		
Acetone	31.26	31.47	31.50	31.41	0.13
DMAc	38.12	38.63	38.52	38.42	0.27
(1:2)	36.91	37.08	37.24	37.08	0.17
(1:1)	35.71	35.61	35.48	35.60	0.12
(2:1)	34.64	34.63	34.79	34.69	0.09

Table 22 First summary of the retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in CA plate from 2:1 acetone-DMAc formulation

Flow rate ml/h	Collection time (minute)	distance (cm)		Spot width (cm)		Retardation time (R_f)		Efficiency (N)		Resolution (R)
		D	P	D	P	D	P	D	P	
0.20	2.00	x	x	x	x					
	4.00	3.20	4.20	0.70	0.60	0.46	0.60	1600.00	2177.78	1.54
	6.00	3.15	4.15	0.70	0.55	0.45	0.59	1600.00	2591.74	1.60
0.40	2.00	x	x	x	x					
	4.00	3.20	4.20	0.70	0.60	0.46	0.60	1600.00	2177.78	1.54
	6.00	3.10	4.10	0.70	0.60	0.44	0.59	1600.00	2177.78	1.54
0.60	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
0.80	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
1.00	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					

Table 23 Second summary of the retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in CA plate from 2:1 acetone-DMAc formulation

Flow rate ml/h	Collection time (minute)	distance (cm)		Spot width (cm)		Retardation time (R_f)		Efficiency (N)		Resolution (R)
		D	P	D	P	D	P	D	P	
0.20	2.00	x	x	x	x					
	4.00	3.10	3.90	0.70	0.60	0.44	0.56	1600.00	2177.78	1.23
	6.00	3.00	3.85	0.65	0.55	0.43	0.55	1855.62	2591.74	1.42
0.40	2.00	x	x	x	x					
	4.00	3.30	4.20	0.68	0.59	0.47	0.60	1695.50	2252.23	1.42
	6.00	3.00	4.00	0.70	0.60	0.43	0.57	1600.00	2177.78	1.54
0.60	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
0.80	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
1.00	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					

Table 24 Third summary of the retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in CA plate from 2:1 acetone-DMAc formulation

Flow rate ml/h	Collection time (minute)	distance (cm)		Spot width (cm)		Retardation time (R_f)		Efficiency (N)		Resolution (R)
		D	P	D	P	D	P	D	P	
0.20	2.00	x	x	x	x					
	4.00	3.10	4.20	0.70	0.60	0.44	0.60	1600.00	2177.78	1.69
	6.00	3.00	4.10	0.70	0.55	0.43	0.59	1600.00	2591.74	1.76
0.40	2.00	x	x	x	x					
	4.00	3.15	4.20	0.68	0.65	0.45	0.60	1695.50	1855.62	1.58
	6.00	3.20	4.20	0.65	0.60	0.46	0.60	1855.62	2177.78	1.60
0.60	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
0.80	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
1.00	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					

Table 25 First summary of the retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in CA plate from 1:1 acetone-DMAc formulation

Flow rate ml/h	Collection time (minute)	distance (cm)		Spot width (cm)		Retardation time (R_f)		Efficiency (N)		Resolution (R)
		D	P	D	P	D	P	D	P	
0.20	2.00	x	x	x	x					
	4.00	x	x	x	x					
	6.00	x	x	x	x					
0.40	2.00	x	x	x	x					
	4.00	2.40	3.50	0.55	0.60	0.34	0.50	2591.74	2177.78	1.91
	6.00	2.35	3.40	0.50	0.55	0.34	0.49	3136.00	2591.74	2.00
0.60	2.00	2.90	4.20	0.90	0.70	0.41	0.60	967.90	1600.00	1.63
	4.00	2.60	3.90	0.70	0.60	0.37	0.56	1600.00	2177.78	2.00
	6.00	2.55	3.90	0.60	0.60	0.36	0.56	2177.78	2177.78	2.25
0.80	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
1.00	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					

Table 26 Second summary of the retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in CA plate from 1:1 acetone-DMAc formulation

Flow rate ml/h	Collection time (minute)	distance (cm)		Spot width (cm)		Retardation time (R_f)		Efficiency (N)		Resolution (R)
		D	P	D	P	D	P	D	P	
0.20	2.00	x	x	x	x					
	4.00	x	x	x	x					
	6.00	x	x	x	x					
0.40	2.00	x	x	x	x					
	4.00	2.30	3.50	0.55	0.65	0.33	0.50	2591.74	1855.62	2.00
	6.00	2.20	3.40	0.55	0.60	0.31	0.49	2591.74	2177.78	2.09
0.60	2.00	3.00	4.30	0.85	0.65	0.43	0.61	1085.12	1855.62	1.73
	4.00	2.50	3.80	0.70	0.60	0.36	0.54	1600.00	2177.78	2.00
	6.00	2.60	3.90	0.60	0.60	0.37	0.56	2177.78	2177.78	2.17
0.80	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
1.00	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					

Table 27 Third summary of the retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in CA plate from 1:1 acetone-DMAc formulation

Flow rate ml/h	Collection time (minute)	distance (cm) D	distance (cm) P	Spot width (cm) D	Spot width (cm) P	Retardation time (R_f) D	Retardation time (R_f) P	Efficiency (N) D	Efficiency (N) P	Resolution (R)
0.20	2.00	x	x	x	x					
	4.00	x	x	x	x					
	6.00	x	x	x	x					
0.40	2.00	x	x	x	x					
	4.00	2.40	3.45	0.55	0.65	0.34	0.49	2591.74	1855.62	1.75
	6.00	2.30	3.30	0.50	0.60	0.33	0.47	3136.00	2177.78	1.82
0.60	2.00	2.80	4.10	0.90	0.75	0.40	0.59	967.90	1393.78	1.58
	4.00	2.80	4.00	0.60	0.60	0.40	0.57	2177.78	2177.78	2.00
	6.00	2.60	3.90	0.60	0.60	0.37	0.56	2177.78	2177.78	2.17
0.80	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
1.00	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					

Table 28 First summary of the retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in CA plate from 1:2 acetone-DMAc formulation

Flow rate ml/h	Collection time (minute)	distance (cm) D	distance (cm) P	Spot width (cm) D	Spot width (cm) P	Retardation time (R_f) D	Retardation time (R_f) P	Efficiency (N) D	Efficiency (N) P	Resolution (R)
0.20	2.00	x	x	x	x					
	4.00	x	x	x	x					
	6.00	x	x	x	x					
0.40	2.00	x	x	x	x					
	4.00	x	x	x	x					
	6.00	2.20	3.20	0.55	0.60	0.31	0.46	2591.74	2177.78	1.74
0.60	2.00	no	no	no	no					
	4.00	2.10	3.10	0.65	0.55	0.30	0.44	1855.62	2591.74	1.67
	6.00	1.40	2.30	0.60	0.50	0.20	0.33	2177.78	3136.00	1.64
0.80	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
1.00	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					

Table 29 Second summary of the retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in CA plate from 1:2 acetone-DMAc formulation

Flow rate ml/h	Collection time (minute)	distance (cm) D	distance (cm) P	Spot width (cm) D	Spot width (cm) P	Retardation time (R_f) D	Retardation time (R_f) P	Efficiency (N) D	Efficiency (N) P	Resolution (R)
0.20	2.00	x	x	x	x					
	4.00	x	x	x	x					
	6.00	x	x	x	x					
0.40	2.00	x	x	x	x					
	4.00	x	x	x	x					
	6.00	2.20	3.15	0.55	0.60	0.31	0.45	2591.74	2177.78	1.65
0.60	2.00	x	x	x	x					
	4.00	2.30	3.20	0.60	0.55	0.33	0.46	2177.78	2591.74	1.57
	6.00	1.45	2.35	0.60	0.50	0.21	0.34	2177.78	3136.00	1.64
0.80	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
1.00	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					

Table 30 Third summary of the retardation factors (R_f), efficiency (N) and resolution (R) of steroid compounds examined in CA plate from 1:2 acetone-DMAc formulation

Flow rate ml/h	Collection time (minute)	distance (cm) D	distance (cm) P	Spot width (cm) D	Spot width (cm) P	Retardation time (R_f) D	Retardation time (R_f) P	Efficiency (N) D	Efficiency (N) P	Resolution (R)
0.20	2.00	x	x	x	x					
	4.00	x	x	x	x					
	6.00	x	x	x	x					
0.40	2.00	x	x	x	x					
	4.00	x	x	x	x					
	6.00	2.20	3.25	0.55	0.55	0.31	0.46	2591.74	2591.74	1.91
0.60	2.00	x	x	x	x					
	4.00	2.35	3.30	0.55	0.55	0.34	0.47	2591.74	2591.74	1.73
	6.00	1.45	2.35	0.55	0.50	0.21	0.34	2591.74	3136.00	1.71
0.80	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					
1.00	2.00	tail	tail	tail	tail					
	4.00	tail	tail	tail	tail					
	6.00	tail	tail	tail	tail					

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