Liquid Chromatography in Pharmaceutical Analytics

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Abstract

This work is discussing the liquid chromatography (LC) in pharmaceutical analytics. High performance liquid chromatography (HPLC) is the popular technique for pharmaceutical analyses, like for example assay and impurities determination. There are many HPLC types, from which reversed-phase chromatography is the most widely used in pharmaceutical analyses.

Fast liquid chromatography is discussed in this literary part. Methods can be speeded up by using appropriates columns. The conventional HPLC method is possible to be speeded up using short columns or columns with small particle packing. The most effective way is to use the core-shell column. If the ultra-high performance liquid chromatography (UHPLC) technique is available, most of the conventional LC methods can be transferred to extra fast separation LC methods without losing required characteristics in pharmaceutical analysis.

Work presents several detectors that are possible to be coupled with the liquid chromatography. The common detectors are UV/Vis detectors and photodiode array detectors (PDA). Work focuses on the aerosol-based detectors and comparison of their performance because these detectors are universal: the giving response is for any analyte, not only for a certain class of compounds. The aerosol-based detectors are charged aerosol detector (CAD), evaporative light scattering detector (ELSD) and condensation nucleation light scattering detector CNLSD (nano quantity analyte detector, NQAD). Many literature examples of the detector performance are presented.
Each aerosol-based detector is practical solution for detection of universal compounds and each one detected all pharmaceutical compounds with sufficient accuracy and sensitivity in the discussed studies. However, CAD and CNLSD/NQAD have better characteristic results, like accuracy and sensitivity than ELSD. But, CAD may be easier to operate than CNLSD/NQAD, which may be complex to operate and it may have tendency to analyte dependent.

Tiivistelmä

Tutkielmassa perehdytään nestekromatografian käyttöön lääkeaineanalytiikassa. Korkean erotuskyvyn nestekromatografia (HPLC) on suosittu menetelmä lääkeaineiden analysoinneissa, esimerkiksi pitoisuuksien tai epäpuhtauksien määrityksissä. HPLC-menetelmät voidaan luokitella useisiin alalajeihin ja käänteisfaasinestekromatografia (RP-HPLC) on niistä suosituin lääkeaineanalytiikassa.

Tässä kirjallisuuskatsauksessa käsitellään erityisesti nopeaa nestekromatografiaa. Analyysimenetelmiä voidaan nopeuttaa sopivan kolonnin avulla. Tavanomainen HPLC-ajo on mahdollista toteuttaa lyhyemmässä ajassa käyttämällä lyhyttä kolonnia tai kolonnia, jossa partikkelikoko on pieni. Kuitenkin tehokkain tapa on käyttää kiinteäytimistä kolonnia. Jos ultrakorkean erotuskyvyn nestekromatografian (UHPLC) vaatimat laitteistot ovat saatavilla, niin useimmat tavanomaiset HPLC-menetelmät voidaan muuntaa UHPLC-menetelmiksi eivätkä lääkeaineanalyysien laatuvaatimukset kärsi.

Työssä esitellään useita detektoreita, joita voidaan käyttää yhdessä HPLC:n kanssa. Ultraviolettisäteilyn (UV) absorption mittaamiseen käytetyt UV/Vis- ja fotodiodirividetektorit (PDA) ovat yleisimmin käytettyjä detektoreita. Tutkielmassa kuitenkin keskitytään tarkastelemaan ja vertailemaan aerosolipohjaisia detektoreita johtuen niiden universaalista luonteesta; ne saavat aikaan vasteen mistä tahansa yhdisteestä. Kirjallisuuskatsauksessa esitellyt aerosolipohjaiset detektorit ovat varautuneen aerosolin detektori (CAD), haihduttava valonsirontadetektori (ELSD) sekä kondensaationukleaatio valonsirontadetektori (CNLSD). Työssä esitellään useita kirjallisuusesimerkkejä aerosolipohjaisten detektoreiden käytöstä lääkeaineanalytiikassa. Kirjallisuuslähteiden perusteella detektorit kykenivät havaitsemaan lääkeaineyhdisteet riittävällä tarkkuudella ja herkkyydellä. Kuitenkin käytettäessä CAD- ja CNLSD-detektoreita saatiin parempia tuloksia kuin käytettäessä ELSD-detektoria. Toisaalta kirjallisuuden mukaan CNLSD-detektori saattaa olla monimutkainen käyttää ja sillä saadut tulokset riippuvat analyytistä, joten CAD-detektori on usein parempi vaihtoehto lääkeaineiden analytiikassa.

Preface

Thesis was made in collaboration between Fermion Oy and University of Jyväskylä. This work contains the literary part and it was done from November 2016 to June 2017. The experimental part was done as the separate document because it was performed as a confidential report. The experimental part was performed from July 2016 to October 2016. The supervisors of the thesis were Laboratory Manager Kaisa Koli (Fermion Oy) and Laboratory Engineer Hannu Pakkanen (Department of Biological and Environmental Science; Department of Chemistry). The scientific publications were searched using Web of Science, ScienceDirect and Google Scholar databases and some of cited books were looked for using search tool of library of University of Jyväskylä.

I would like to thank specially my supervisors Kaisa Koli and Hannu Pakkanen for offering the helpful advices and interesting topic in the area of pharmaceutical analytics, which has interested me a lot during my studies. I would also like to thank the whole team in Fermion Oy's Analytical Development and Quality Control laboratory who supported and helped me during my experimental project. Also, great thanks to my family and friends for the encouragement.

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Annika Sneck

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Abbreviations

ACN	Acetonitrile
API	Active Pharmaceutical Ingredient
BEH	Ethylene Bridget Hybrid
CAD	Charged Aerosol Detector
CLA	Clarithromycin
CN	Condensation Nucleation
CNLSD	Condensation Nucleation Light Scattering Detector
DAD	Diode Array Detector
DCM	Dichloromethane
EAA	Electrical Aerosol Analyzer
ECD	Electrochemical Detector
ELSD	Evaporative Light Scattering Detector
EMEA or EMA	European Medicines Agency
ERY	Erythromycin
FDA	US Food and Drug Administration
FIA	Flow Injection Analysis
GMP	Good Manufacturing Practice
HETP	Height Equivalent to the Theoretical Plate
HPLC	High Performance Liquid Chromatography
HTLC	High Temperature Liquid Chromatography
ICH	International Conference on Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use
IEC or IEX	Ion-Exchange Chromatography
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
NQAD	Nano Quantity Analyte Detector
NP-HPLC	Normal-Phase Chromatography
OLE	Oleandomycin
PDA	Photodiode Array Detector
Ph.Eur	European Pharmacopoeia

PMT	Photomultiplier Tube
ppm	Parts per million
RI	Refractive Index
ROX	Roxithromycin
RP-HPLC	Reversed-Phase Chromatography
RRF	Relative Response Factor
RSD	Relative Standard Deviation
RT	Retention Time
SD	Standard Deviation
SEC	Size-Exclusion Chromatography
S/N	Signal-to-Noise Ratio
SOP	Standard Operating Procedure
TEA	Triethanolamine
TFA	Trifluoroacetic Acid
TRO	Troleandomycin
UHPLC	Ultra-High Performance Liquid Chromatography
USP	United States Pharmacopeia
UV/Vis	Ultraviolet/Visible

1 Introduction

High performance liquid chromatography or HPLC has become very popular analytical method in many areas in pharmaceuticals, including product separation and purification, over the last few decades.[1] HPLC is also widely used in biotechnology and environmental monitoring. “The advance and availability of HPLC columns of different geometries and various stationary phases have been counterpointed by the development of reliable and rugged HPLC equipment, the performance of which is now compatible with that of the extremely efficient and modern columns.”

This thesis concentrates on the applications of HPLC and is also dealing with ultra-high performance liquid chromatography (UHPLC) in pharmaceutical analytics. First the general about HPLC system, including the instrument is presented and then theoretical considerations, as chromatographic parameters and related theories are discussed. It is followed by discussion about separation techniques, like reversed-phase chromatography. Second, the drug analysis by LC are dealt with including column selection and method transfer. Third, common detectors and primarily aerosol-based detectors are presented that are possible to couple with LC. Many literature examples of the detector performance are discussed. Final part of the thesis focuses on the method validation and the literature example of pharmaceutical cleaning validation is presented.

2 Liquid Chromatography

2.1 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a method for controlling purity of drugs or new drug candidates, monitoring changes in synthesis and performing quality control or assurance of the final drug products.[2] HPLC is actually primarily used in drug industry for evaluations of a large variety of samples. LC is an analytical technique in which the components of a mixture solution are separated on stationary phase which located in a column.[3] The flowing system which is called the mobile phase enables the separation. The mobile phase is a liquid when we are talking about liquid chromatography (LC). The separation occurs since each component in a mixture will interact with the two phases variously relative to the other components in the mixture and moreover the separation requires an optimum set of conditions to occur.

Chromatographic separation can be applied for compounds that migrate at different rates through a "chromatographic bed".[1] The occurrence of different migration speeds is based on different retentions of the migrating compounds, that are called analytes. Different retentions produced by process of different distributions of the analytes between two phases: a mobile phase and a stationary phase. The best separation mechanism and column characteristic can be chosen for a certain problem.[3] This is based on the nature of the components in the mixture together with the chemical and physical characteristics of the column.

In an HPLC system the liquid mobile phase is mechanically pumped through a column that includes the stationary phase. An HPLC instrument comprises a solvent reservoir, an injector, a pump, a column, a detector and a data processing unit (Figure 1).[3]

A solvent reservoir enables storage of sufficient amount of solvent for continual operation of the HPLC system.[4] A pump makes possible the constant and non-stop flow of the mobile phase through the system. Often the pumps allow mixing of solvents from different reservoirs. An injector allows an introduction, or injection of the mixture (analytes) into the stream of the mobile phase and then it enters the column. Commonly modern injectors are autosamplers. A column produces a separation of the analytes in the mixture. There is the mobile phase in contact with stationary phase in the column. In the recent years, most of chromatographic development have focused on the column and ways to enhance this interactive contact between mobile and stationary phases. A detector monitors the response, which is due to physical or chemical properties of the column effluent. The most common detector is UV (ultraviolet) detector used in pharmaceutical analysis. It monitors the UV absorbance at a defined wavelength or over a wide range of wavelengths (diode array detector, DAD). There are alternatives to the common detectors, for example aerosol-based detectors. A data processing unit (control system and data acquisition) is computer-based system for controlling parameters of HPLC system, for example eluent composition, temperature or injection sequence, and obtaining data from the detector and monitors system performance, for instance temperature, backpressure etc.

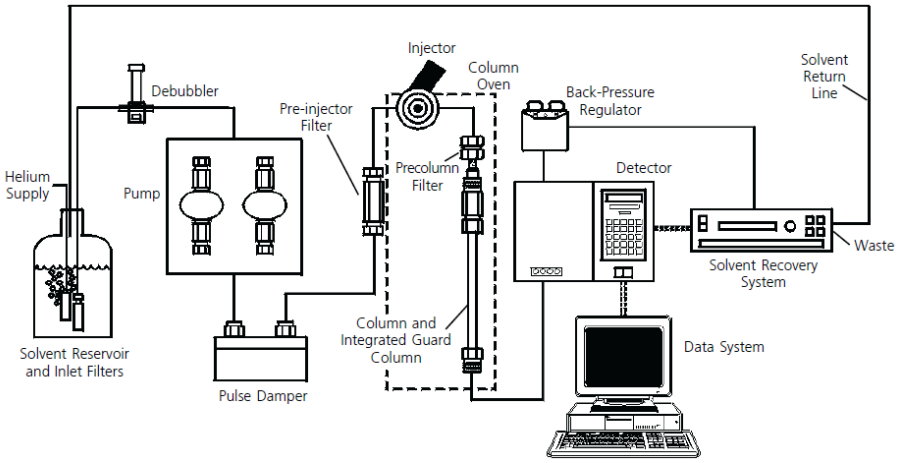

Figure 1. Components of an HPLC system.[5]

It is very important that modern HPLC method development includes the following considerations: developed method would resolve potential impurities and degradation products, developed strategies for instrument qualification meet regulative requirements and official validation of HPLC method is performed before it is utilized routinely.[6]

2.2 Chromatographic Parameters

Detectability optimization and selectivity are the primary targets in HPLC separations.[6] Backgrounds of these characteristics are two theoretical main aspects: kinetic aspect of chromatographic zone migration is responsible for band broadening and thermodynamic aspect is responsible for the analyte retention in the column. The procedure of chromatographic zone dispersion in the column is usually called efficiency. It is one of the major descriptors of chromatographic system. The velocity of chromatographic zone migration is defined by the analyte competitive interreactions with the stationary phase. The rate is constant in the isocratic separation mode. Retention time (RT) is the general analyte retention characteristic, and RT is the time that analyte reaches the detector.

Major basic chromatographic parameters are four and they are commonly used to report characteristics of the chromatographic column, system and specific separation: retention factor (*k)* which refers to capacity, efficiency *(N)*, selectivity (α) and resolution (*R*).[6] The capacity and selectivity of the column are variables that are controlled by the column manufacturer, and efficiency and resolution can be controlled by the user of chromatography (in some extent).[3] To

acquire the best possible separation, the efficiency of the system must be optimized in order to minimize band broadening.

2.2.1 Retention Factor

Retention factor (or capacity factor) is measuring the retention of the certain compound in a certain chromatographic system.[6] It is defined as:

$$k = \frac{V_R - V_0}{V_0} = \frac{t_R - t_0}{t_0} \quad (1)$$

where V_R is the analyte retention volume (volume of solution that is pumped through the detector before specific peak is eluted), V_0 is the volume of the liquid phase (void volume, volume of solvent pumped through the detector between the time of injection and the appearance of the non-retained components), t_R is the analyte retention time (time taken for a particular solute to reach the detector) and t_0 defined as the retention time of non-retained analytes (holdup time, time taken for non-retained species to reach the detector). The void volume is equal to the volume of the column that is not taken by packing material. Capacity factor is a function of the packing material of a column.[3] When the capacity factor of a column is higher, its ability to retain solutes is greater. A higher retention factor can improve the resolution of a separation. On the other hand, a higher retention factor increases the analysis time. Values of retention factor between 1 and 10 are usually acceptable, but typically a great balance of analysis time and resolution is caused by values between 2 and 5. Retention factor is independent of mobile phase flow rate and the column dimensions. All other chromatographic conditions influence analyte retention.

Chromatographic separation is based on a continual sequence of equilibrium distribution.[1] It induces the compounds to migrate at different rates through the chromatographic bed and their migration speed is presented as follows:

$$u_i = u_0 \left(\frac{1}{1+k_i}\right) \quad (2)$$

where u_0 is the mean migration speed of the mobile phase components and k_i is the retention factor (Equation 1).

2.2.2 Efficiency

Efficiency is the degree of peak dispersion in a specific column.[3] The phenomenon called band broadening is linked to the efficiency and it can be described as follows: The band width of the sample is very narrow when a sample mixture is only applied to the head of a column. The widths have broadened by the time when the components are eluted from the end of the column. The phenomenon occurs because the various sample components interact with the stationary phase and they are retained varied degrees in the stationary phase. The components travel through the packing material of the column and this interaction causes a process called band broadening which means the increase in band width. The degree in which two components can be separated is determined by the amount of band broadening, thus band broadening should be a minimum. Two theories have developed to describe efficiency of a column and they are called the plate theory and the rate theory, that will be covered in the next sections.

2.2.2.1 Plate Theory

The plate theory was proposed by Martin and Synge[7] and it has been developed to provide a convenient way to measure the efficiency and the performance of column.[3] Efficiency of a column is the measure of the amount of peak broadening as a function of retention, and it denotes as the number of theoretical plates (N) calculated as:

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

where t_R is the analyte retention time and w is the peak width at the baseline.[6] Efficiency is essentially the characteristic of the column. According to the plate theory the chromatographic column is viewed to comprise of a number of thin sections or "plates" which allows a solute to equilibrate between the mobile and stationary phases.[3] When the number of theoretical plates is greater the more efficient is the column. Plate theory supposes that the solute between the mobile and stationary phase is in instantaneous equilibrium and it doesn't treat the effects of diffusional effects on performance of column. Efficiency N will be treated in more specific way in the next paragraphs.

Molecular diffusion is the only parameter of kinetic aspect, if all specific processes, for example analyte ionization and surface interactions have fast rate constants and thermodynamic equilibrium.[3,6,7] Column properties can be perceived isotropic, and then symmetrical peaks of a

Gaussian shape is expected (Figure 2). The variance of the peak is proportional to the diffusion coefficient:

$$\sigma^2 = 2Dt \qquad (4)$$

The substance moves through the column with length (L) during the time (t) at given linear velocity (v):

$$L = vt \qquad (5)$$

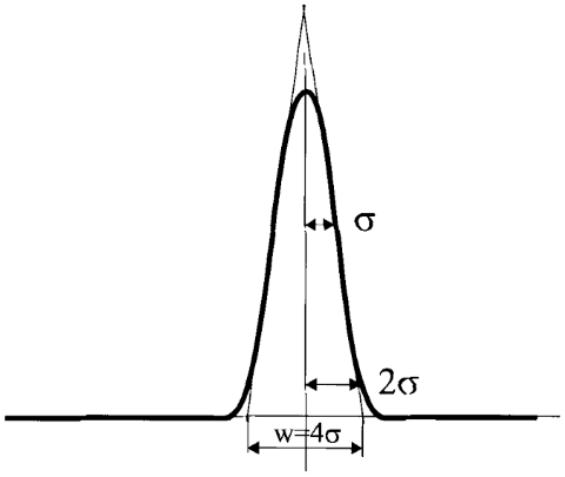

Figure 2. Chromatographic peak with symmetrical Gaussian band broadening.[6]

Replacing *t* from Equation (5) in Equation (4) the following expression is obtained:

$$\sigma^2 = \left(\frac{2D}{v}\right) L \qquad (6)$$

where *2 D/v* is essentially the measure of band broadening at certain velocity at the distance *L* of the column.[3,6,7] It is, in essence, the height equivalent to the theoretical plate and could be denoted as *H* (or HETP)*:*

$$H = \frac{\sigma^2}{L} \qquad (7)$$

There are many different processes leading to the band broadening phenomena in the column. They are for example molecular diffusion, multipath effect, displacement in the porous beds and secondary equilibria. Every previous process adds its own degree of variance in the total band broadening process. Total band broadening is equal to the sum of the variances for every independent process based on the fundamental statistical law:

$$\sigma_{tot}^2 = \sum \sigma_i^2 \qquad (8)$$

In the Equation (8) the definition of *H* is identical to the plate height as it evolved from the distillation theory and was brought to modern chromatography.

As *H* the theoretical plate height can be determined the overall number of the theoretical plates in the column as follows:

$$N = \frac{L}{H} \rightarrow N = \left(\frac{L}{\sigma}\right)^2 \quad (9)$$

Thus, the greater is the efficiency of the column, the smaller is the height equivalent to a theoretical plate (HETP).[3,6] The H is smaller for small stationary phase particle sizes, less viscous mobile phases, low mobile phase flow velocities, smaller solute molecule sizes and higher separation temperatures.

Every analyte moves through the column with constant velocity (u_c) in linear chromatography.[6] The analyte retention time can be expressed using this velocity:

$$t_R = \frac{L}{u_c} \quad (10)$$

The required time can be determined to travel the analyte zone through the column a distance of one σ (Figure 2) as τ:

$$\tau = \frac{\sigma}{u_c} \quad (11)$$

Replacing Equations (10) and (11) into Equation (9) is obtained:

$$N = \left(\frac{t_R}{\tau}\right)^2 \quad (12)$$

In the previous Equation (12) parameter τ is the fraction of peak width equivalent to the standard deviation σ. Because symmetrical band broadening of a Gaussian shape being considered, the Gaussian function can be used to relate its standard deviation to more easily measurable quantities. The most generally used points are the so called peak width at the baseline which is in reality the distance between the points of intersections of the tangents to the peaks inflection points with the baseline (Figure 2). The distance is equal to the four standard deviations and thus the final equation for the efficiency will be:

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

Efficiency is chiefly a column-specific parameter. In HPLC the variations of the flow rate of mobile phase do not affect column efficiency exceedingly within the applicable flow rate region.

Density and uniformity of the column packing and geometry of the packing material are the prime factors defining the efficiency of a certain column. There is no explicit relationship between the particle diameter and the predictable column efficiency. At the same time an increase of the efficiency can be expected to occur with a decrease of the particle diameter, because there is a decrease of the gap between the average pore size in the packing material and the effective size of interparticle pores. It leads to a more equal flow around and inside the particles. The smaller is the particles, the lower is the theoretical plate height and the higher is the efficiency (Figure 3).

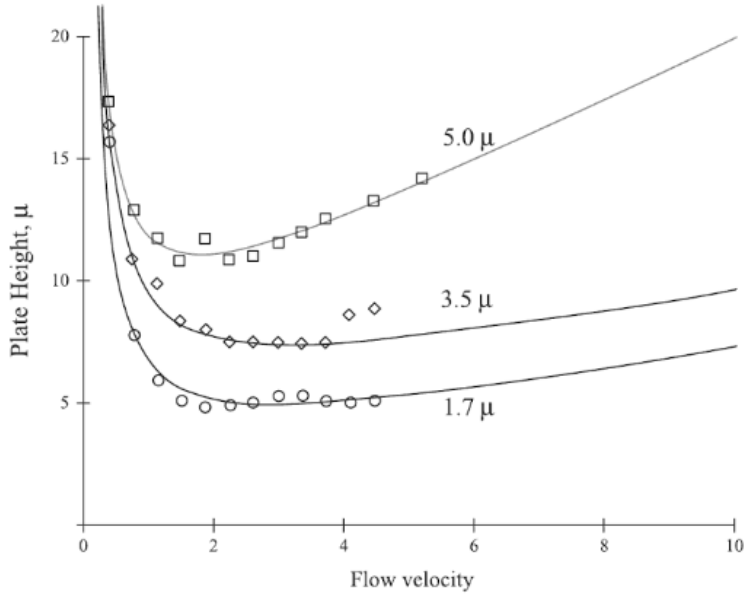

Figure 3. The dependence of the theoretical plate height (HETP) on the flow velocity for columns packed with particles of different diameter (5.0 μm squares; 3.5 μm rhombs; 1.7 μm circles).[4]

2.2.2.2 Rate Theory and Band Broadening

Rate theory by Van Deemter *et al.*[8] provides ways to measure contributions (diffusional factors) to band broadening and so optimize the efficiency in the column.[3,6] Rate theory doesn't assume instantaneous equilibrium such as plate theory does. The universal form of the shown dependence (Figure 3) is known as the Van Deemter function, which is presented as follows:

$$H = A + \frac{B}{v} + C_v \qquad (13)$$

where H signifies the efficiency of the column, v is the linear flow velocity of the mobile phase and A, B and C are constants for certain column and mobile phase. They signify three different processes that contribute to the total chromatographic band broadening:

A: Multipath effect or eddy diffusion

B: Molecular diffusion

C: Mass-transfer

The multipath effect A is a flow self-contained term which determines the ability of different molecules to move through the porous media with paths of different length, so it represents the contribution to band broadening by eddy diffusion. The molecular diffusion term B is inversely proportional to the flow velocity which denotes that the slower flow rate, the longer the component stays in the column. Then the molecular diffusion process has more time to broaden the peak. Therefore, B represents the contribution from longitudinal diffusion. The mass-transfer term C is

equivalent to the flow velocity. The flow being faster, the band broadening is the higher because of larger lag between a retained molecule compared to a molecule traveling with the flow. That is *C* represents the contribution from resistance to mass transfer. Schematically superposition of processes is presented in Figure 4.

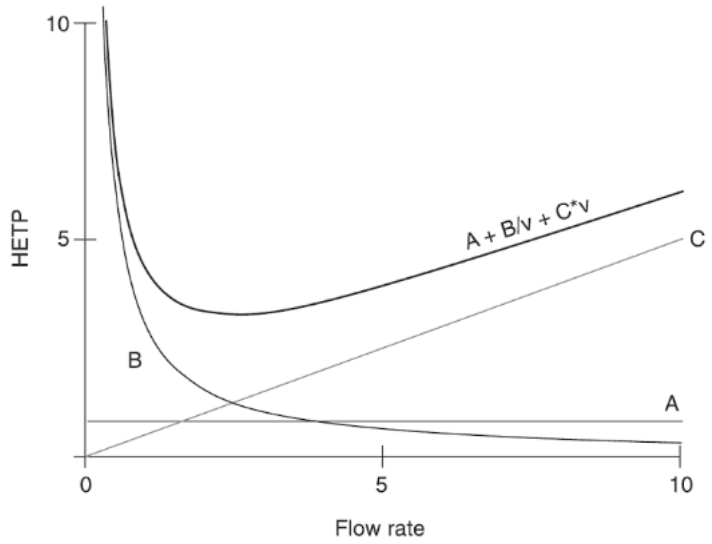

Figure 4. Schematic of the Van Deemter function and its components.[4]

Dependencies of the column efficiency on the flow rate follow the Van Deemter curve, which is a theoretical function (Figures 3 and 4).[6] In theory, there is an optimum flow speed that causes the highest efficiency, which is equivalent to the lowest theoretical plate height. When the particle diameter is lower, the range of flow velocities is wider where the highest column efficiency is accomplished (Figure 3). Columns that have been packed with small particles can function with high efficiency being fast flow velocity since these columns have low mass-transfer term *C*. In that case retention equilibrium is attained faster in these columns.

Faster flow velocities denote higher flow resistance and higher backpressure.[6] It is a present-day trend to work with the small particles at high linear velocity. However, the total efficiency of the columns packed with smaller particles is not much higher. The ability to increase *u* depends on the pressure capabilities of the equipment because pressure is proportional to velocity:

$$\Delta P = \frac{uL\eta\phi}{d_p^2} \qquad (14)$$

where ΔP is the pressure drop across the column, η is the viscosity and ϕ the flow resistance factor. The fastest possible separation requires that the maximum pressure allowed by the equipment be used. In that case supposing the resolution requirement is qualified. This also denotes the rate of

analysis is limited by maximum pressure. As short column as possible should be used to limit analysis time. The pressure drop is another reason to limit column length. Shorter columns have lower pressure requirements and they allow the use of the pressure to gain an advantage in rapidity. *N* will decrease as *u* increases, which means that at faster velocities longer columns are essential to give the required theoretical plates thus requiring greater pressures.

2.2.3 Selectivity

Selectivity is the ability of chromatographic system to separate two different analytes and it is determined as the ratio of corresponding capacity factors, or retention times or volumes between to certain peaks.[6] Selectivity is usually defined in terms of α:

$$\alpha = \frac{k_2}{k_1} = \frac{t_2 - t_0}{t_1 - t_0} = \frac{V_2 - V_0}{V_1 - V_0} \qquad (15)$$

The selectivity of a column is mainly a function of the packing material, but the user of chromatography has some control of that modifying the mobile phase or temperature.[3] The value of α is unity (1) if the retention times of the two components are the same (t_2=t_1) and then separation will not occur. α can range from unity to infinity if the first component is eluted in the void volume. Changing the composition of mobile phase is a good approach to increase α. If changing the concentration of the one component is not sufficient, usually modifying the nature of one of the components will often be a practical approach to increase α.

2.2.4 Resolution

Resolution as term describes the degree of separation between adjacent solute bands or peaks and it is determined as:

$$R = 2\frac{t_2 - t_1}{w_2 + w_1} \qquad (16)$$

Resolution is qualified as the ability of column to resolve two analytes into separate peaks.[6] Also, the resolution is defined as half of the distance between the centers of gravity of two chromatographic zones related to the sum of their standard deviations as follows:

$$R = \frac{X_2 - X_1}{2(\sigma_1 + \sigma_2)} \qquad (17)$$

In instance of symmetrical peaks, centers of peak gravity can be replaced with the peak maximum and using the relationship between Gaussian peak width and its standard deviation.[6] In that case is obtained the following expression:

$$R = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(w_2 + w_1)} \quad (18)$$

If Equation (3) is used for replacement of the peak width in Equation (18) then the following equation is obtained:

$$R = \frac{t_{R2} - t_{R1}}{t_{R2} + t_{R1}} \frac{\sqrt{N}}{2} \quad (19)$$

Equation (19) shows that the resolution is proportional to the square root of the efficiency.

Another approach to present the resolution equation: resolution is affected by the selectivity (α), efficiency (N) and capacity (k) of the column:

$$R = \frac{1}{4} \frac{\alpha - 1}{\alpha} \sqrt{N} \frac{k}{1 + k} \quad (20)$$

The Equation (20) expresses the relationship between those factors and shows possibility to control them in order to improve the resolution between two peaks.[3] Changing the selectivity or retention factor of the column is the most efficient way to modify the resolution. Increasing the retention factor will increase the analysis time, however. Some compromise between them must be attained.

There is one more option to improve the resolution; increasing the efficiency.[3,6] The efficiency is proportional to the column length and it means the longer the column the higher the efficiency. Secondly Equations (19) and (20) demonstrates that the increase of the efficiency adds the resolution only as a square root function (Figure 5).

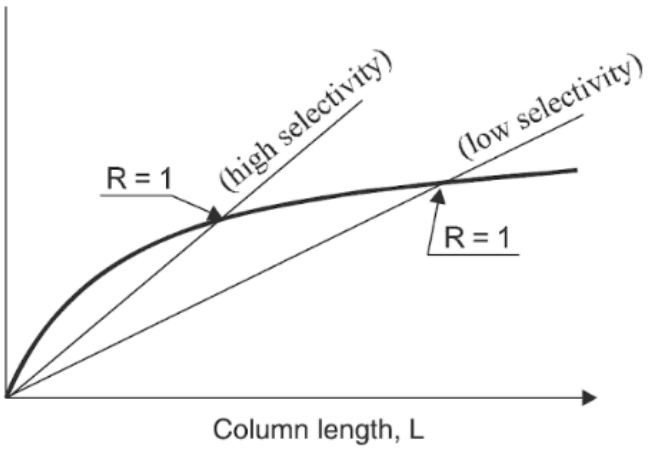

Figure 5. Relationship between resolution, selectivity and column length.[4]

On the other hand, an increase of the column length results in an increase of the flow resistance and backpressure, which limit the total column length.[6] At low selectivity, to attain the same resolution, a longer column must be used to compensating selectivity with higher efficiency and increasing the pressure. The following equation is called Kozeny-Carman equation which is expressed the relationship between column length, viscosity of the mobile phase and the backpressure:

$$\Delta p = \frac{\varphi \eta \langle v \rangle L}{d_p^2} \qquad (21)$$

where φ is an empirical coefficient conditional on the column porosity and it is usually between 500 and 1000, η is viscosity of the mobile phase, $\langle v \rangle$ is the mobile phase mean flow velocity, L is the length of the column and d_p is the particle diameter of the packing material. The decrease of the particle diameter leads to the quadratic increase of the column backpressure. Equation (14), on the other hand, shows a linear increase of the backpressure with an increase of the flow velocity, viscosity of the mobile phase and length of the column.

2.3 Separation

A variety of HPLC separation mechanism are based on various criteria such as the nature of the stationary and mobile phases and the type of interactions supposed to lead to the separation.[9] Also the range of concentration of specific solvents in the mobile phase, for example of water, is impacted. Different HPLC types have different characteristics and applications and understanding of these differences is significant, thus it is easier to select the relevant HPLC type for solving the exact analysis problem or separation.

The main types of HPLC techniques are reversed-phase chromatography (RP-HPLC), normal-phase chromatography (NP-HPLC), ion-exchange chromatography (IEX) and size-exclusion chromatography (SEC) in pharmaceutical industry.[4] The dominant type of molecular interactions employed (ionic, polar or dispersive forces) is the principal characteristic that defines the identity of each technique: dispersive forces (hydrophobic or Van der Waals interactions) are the dominant type of molecular interactions employed in RP-HPLC, polar forces employed in NP-HPLC and ionic forces in IEX. There is no force employed in SEC, in other words it is based on the absence of any specific analyte interactions with the stationary phase.

2.3.1 Reversed-Phase Chromatography

Reversed-phase chromatography is the most common HPLC technique and a very large number of compounds can be separated using this type of chromatography.[9] The mobile phase is a polar component and stationary phase is a nonpolar component in RP-HPLC. The stationary phase in the column can be for example chemically bonding long hydrocarbon chain on a solid surface like silica. The most common chain bound to silica is C18 (contains 18 C-atoms) and it has a high hydrophobic character. Polymeric materials are also used as the stationary phase. The mobile phase is primarily a mixture of an organic solvent and water, and buffers are an essential part of the mobile phase, too. pH of the buffer is selected based on the pKa of the analyte (+/- 2 pH rule) in order to control the ionization of the analyte. The interactions are viewed to be the hydrophobic forces and they are followed from the energies resulting from the disturbance of the dipolar structure of the solvent. The solvophobic effect results from the force of "cavity-reduction" in water around the analyte and the nonpolar stationary phase when they are interacting. The retention of the analyte on the stationary phase is dependent on the contact surface area between the stationary phase and the nonpolar moiety of the analyte molecule (aqueous eluent), thus an analyte with a larger hydrophobic surface area is more retained on the stationary phase. So, it has longer retention time compared with an analyte with a smaller hydrophobic area. Most compounds have at least some hydrophobic moiety in the structure thus it is benefit using RP-HPLC. The separation is generally perceived to be based on the partition of the analyte between the stationary phase and the mobile phase but on the other hand some experiments can be explained by adsorption equilibrium.

2.3.2 Normal-Phase Chromatography

Normal-phase chromatography, or NP-HPLC, is a chromatographic type that uses a nonpolar mobile phase and a polar stationary phase for the separation of polar compounds.[9] The nonpolar mobile phases are solvents like dichloromethane (DCM), hexane, heptane or tetrahydrofuran (not water soluble). Commonly there is the small addition of polar modifier, for example methanol or ethanol in nonpolar mobile phase solvent in some cases.[4] The polar modifier additive in the mobile phase (ratio of polar/non-polar mobile phases) allows for the control of the analyte retention in the column. NP-HPLC separation is based on the differences in the strength of the polar interactions of the analytes in the mixture with the stationary phase. The analyte retention is longer if the analyte-stationary phase interaction is stronger. The separation is a competitive process, because analyte molecules compete with the mobile-phase molecules for the adsorption sites on the surface of the

stationary phase. The stronger the mobile-phase interactions with the stationary phase, the lower the difference between the stationary-phase interactions and the analyte interactions, and thus the lower the analyte retention. The most nonpolar compounds elute first and the most polar compounds elute last in NP-HPLC.[9] Packing materials are commonly porous oxides such as silica or alumina.[4] Polarity of surface of these stationary phases is increased with dense population of OH-groups. On the other hand, polarity of silica stationary phases is decreased with trimethoxy glycidoxypropyl silanes (diol-phase). Analyte retention on the surface of the stationary phase is sensitive to the variations of the mobile-phase composition. NP-HPLC uses primarily nonpolar solvents and for that reason it is the method of choice for highly hydrophobic compounds, which are not soluble in polar or aqueous solvents. Thus selection of using this as chromatographic method of choice is generally related to the sample solubility in certain mobile phases. Modifiers of the NP mobile phases based on the structural properties of the analyte: base/acid (TEA/TFA) are commonly used. Also, chiral chromatography (NP-HPLC) is very commonly used in pharmaceutical analysis.

2.3.3 Ion-Exchange Chromatography

Ion-exchange chromatography (IEC) is type of HPLC used for the separation of cations or anions (inorganic or organic). It is founded on the various affinities of the analyte ions in a solution for the oppositely charged ionic centers in the adsorbed or resin counterions in the stationary phase (hydrophobic).[4] Ionic species are retained on the column founded on coulombic interactions.[9] The exchange of two ions A^- and B^+ between the solution and exchange resin E^- is considered as follows:

$$A \cdot E + B^+ \leftrightarrow B \cdot E + A^+$$

And equilibrium constant for the process is presented on the following equation:

$$K = \frac{[A^+][BE]}{[AE][B^+]} \qquad (22)$$

Equation (22) determines the relative affinity of both cations to the exchange centers on the surface.[4] The higher the K (greater than 1), the greater is the ability of B^+ (cation) to substitute A on the surface of resin. The resin can be anion-exchanger if there are positive ionic centers on the surface. Respectively, the resin can be cation-exchanger if there are negative centers on the surface. Material of ion-exchange resin is for example crosslinked styrene-divinylbenzene which exchange groups are attached to the phenyl rings. The material is harder and it is less sensitive to swelling if degree of crosslinkage is higher, but ion-exchange capacity may be lower. Strong cation-exchanger is for example SO_3^-, weak cation-exchanger CO_2^-, strong anion-exchanger quaternary amine and

weak anion-exchanger tertiary amine. Analyte retention and selectivity of this chromatography type are very dependent on the ionic strength and pH of the mobile phase. The mobile phase commonly comprises of buffer solutions.[4,9]

2.3.4 Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) as the method separates the molecules according to their size.[4] The separation is based on the exclusion of the molecules from the porous space of packing material and it is due to their steric hindrance. Determination of analyte retention is founded on the hydrodynamic radius (the main factor) of the analyte molecule, and generally the higher the hydrodynamic radius the shorter the retention and faster elution. SEC is the divergent method for chromatographic separation because there should be avoid any positive interaction of the analyte with the stationary phase. If the interaction happens between analyte molecule and stationary phase, thus the retention of larger molecules increases. It may confound separation based on only hydrodynamic radius of the molecules. SEC determines weight of polymer molecular by defined the relationship of the molecular hydrodynamic radius with the molecular weight, and further it is specified that the logarithm of analyte molecular weight is proportional to the analyte retention volume. They have a linear relationship.

2.4 Ultra-High Performance Liquid Chromatography

Ultra-high performance liquid chromatography or UHPLC is the newish method that is used in addition to HPLC in industrial laboratories.[10] It is often used particularly in the pharmaceutical industry because it has high resolution, speed and sensitivity. Moreover, solvent saving and short runtime are advantageous features. Reduced analysis time is possible by using for example a sub-2 μm column and at the same time mobile phase consumption is small. This had a significant impact on method development scouting time due to shorter runtime. The ultra-high performance of liquid chromatography is possible to achieve either by UHPLC instrumentation which couples with sub-2 μm particle column packing or by conventional HPLC which utilizes core-shell particle column packing. On the other hand, the sub-2 μm column generates a high back pressure and UHPLC system carries very high pressures, even 1,000 bars. The column with core-shell (or fused-core) particle packing causes column back pressure as high as 400 bars. Many existing HPLC methods

are possible to update UHPLC method in pharmaceutical industry, and the other way round. In practice UHPLC method may require to be converted to HPLC if the firstly mentioned system is not available.

The UHPLC system will be presented more accurately in the next chapter.

3 Drug analysis by Liquid Chromatography

3.1 Fast Liquid Chromatography

Faster speed and higher separation efficiency are of interest in liquid chromatography.[10,11] Pharmaceutical industry specially is interested to find fast and efficient methods for analysis, because large number of various samples are analyzed constantly and therefore it is necessary to decrease the individual analysis time. The reduced time required for results and an increase in the separation potential are possible to attain by enhancing the column efficiency. Actually, many pharmaceutical applications, for example quality control, purity assays, pharmacokinetic and medication metabolism studies need high throughput separations.[11] Increasing the mobile phase flow speed and decreasing the length of column are the easiest way to increase the speed of analysis in LC. One solution for the requirement of decrease the analysis time is the use of short columns packed with shell particles and these core-shell packing materials are commercially available in different diameters and various shell thickness. The velocity of analysis can be also increased by using sub-2 μm porous particles and monolith columns. In general, the column technology is evolving much faster than the liquid chromatography instrumentation these days. However there is a need to develop better instruments more with higher upper pressure limit, faster injection cycle time, reduced extra-column variance and lower system dwell volume.

3.2 Column Selection

The column is the main component for the HPLC separation and its selection is very critical for the success of the analysis.[9] It can be difficult to choose the most appropriate analytical system among the all existing options. Many types of columns are commercially available and they can be divided to the five groups due to differences:

1. The nature of the active stationary phase (for example RP-HPLC, IEC, SEC, HILIC etc.)

2. The type of phase (monoliths, porous particles, superficially porous or core-shell particles)
3. Physical characteristics of particles (porosity, dimension, strength etc.)
4. Column dimensions (diameter, length)
5. Mechanical construction (columns, compressible columns, cartridges).

The column selection is performed in accordance with the type of chosen separation, requirements of analysis along with instrument and information availability. The main criterion in the selection of the column is the proper separation and this has to produce narrow peaks without tailing. When the peak shape is good, peak area measurement, peak recognition, data averaging and other data processing operations done by the computer software controlling the HPLC equipment are performed reproducibly and accurately.

The choice depends largely on the properties of the compound to be separated and this is associated with the choice of the mobile phase.[9]

The selection of a column is delineated by the four characteristics:

1. The properties of the separated compounds
2. The specific column properties that are compound independent
3. The requirements of the separation (independent of analytes and mobile phase)
4. The requirements of the mobile phase.

It is important to gain good resolution R values for the compounds in order that the separation will be managed. This is possible to achieve by using columns with large N values and high k and α for the compounds for analysis. In some cases, low t_R (commonly is implied low k values) is essential in a separation when fast analysis is required. An improvement of R can be achieved using shorter and narrower columns and higher flow rates.

The column should be independent of a specific compound and can be considered the number of theoretical plates N.[9] The small and uniform particles or core-shell particles lead to the higher N values. Longer columns also lead to increase of N but the analysis time and column backpressure are increased at the same time. The modification in column diameter also may has an effect to the separation. The larger diameter, the more number of samples can be injected. There is no affect to results but have a little lower N (for the same length). The columns with larger diameter need larger volumes of mobile phase. Moreover, the column should be chemically stable and stand a wide pH range but high backpressure produced by the column is not desirable. Maintaining the equivalent separation characteristics when many samples are analyzed is the significant property, and this will speed up peak identification and data reporting.

The analysis requirements affect also in the choice of column.[9] Analysis time, the number of samples, possible desire to collect the separated analytes and limitations in relation to the nature and the solvent volume to be used in the mobile phase influences the selection of the column. The other effective factors are for example the type of instrumentation or its detector and maximum pressure which the pumps can deliver. Mobile phase properties, for example pH, viscosity and water content may affect the column choice, too.

3.2.1 Sub-2 μm Particles

The columns with very fine particles, sub-2 μm and even sub-1 μm, are developed for the very fast separation, but sensitivity and separation are improved at the cost of pressure.[10-12] Knox and Saleem[13] presented first the approach of compromise between efficiency and speed at the end of 1960s. Decreasing analysis time requires high kinetic efficiency and therefore this denotes that peak widths have to be as narrow as possible. Sensitivity and separation are improved using very fine particles because of narrow peaks, but at the same time pressure is increased. However, most HPLC instruments have a maximum pressure limit of 400 bar thus they are not applicable for coupled with columns that generate very high pressure. Due to the pressure limitation, the system for ultra-high pressure separation has been developed. It is called ultra-high performance liquid chromatography, or UHPLC which is compromise between speed (pressure drop) and efficiency. It allows the very high pressure during analysis in instrument and column. The system for ultra-high pressure separation is not still very old, because the first one was released only in the year of 2004 (Water Acquity UPLC). Nowadays the many UHPLC systems are commercially available and they can work even 1200-1300 bar. In UHPLC system the effect of frictional heating causes temperature gradients within the columns. The radial temperature gradient is a result of the heat dissipation at the column wall and it can cause remarkable loss in plate count. Actually, both longitudinal and radial temperature gradients are more significant when the column length is decreased. Also, the smaller is the diameter of particle, the greater the difficulty is in the quality of column packing in narrow bore columns, because it is harder to prepare a well-packed column bed.

Columns packed with 1.5–2 μm fully porous particles are applied with significant success in pharmaceutical and biomedical analysis.[11,12] Nonporous and porous particles are the major types of spherical packing materials used for fast HPLC and the main difference between them is that porous particles have a resistance to mass transfer contribution from the stagnant mobile phase in the pores. Nonporous particles can enable lower mass transfer resistance and thus higher efficiency than

porous ones. On the other hand, porous particles have larger surface areas and they can provide higher sample loading capacity. Nonporous particles have very low retention compared to porous particles.

3.2.2 Core-Shell Particles

Core-shell particles are made of a solid and nonporous silica core surrounded by a shell of a porous material.[10] The principle of shell type of superficial stationary phase was presented first time by Horváth *et al.*[14] in the 1960s. Fused-core technology was initially developed by Kirkland[15] in the 1990s. Later this technology became commercially available in the year of 2007 under Halo™ from Advanced Materials Technology. Moreover, these sub-3 μm and sub-2 μm shell particles with a highly thin porous layer were published in the year of 2009 and these were 2.6 μm and 1.7 μm Kinetex™ particles from Phenomenex. This Core-Shell™ technology affords particles with a 1.9 μm or 1.24 μm nonporous solid core surrounded by a 0.35 μm or 0.23 μm porous shell layer of silica, respectively.

The advantage of using fused-core particles is that it does not require instrumentation change for UHPLC, but the conventional HPLC instrumentation is possible to use with core-shell particle columns.[16] Additionally, the small columns with core-shell packing can afford faster separations than the columns of same size packed with fully porous particles for both small and macromolecules.[12] Nowadays the core-shell packing material columns are commercially available in the different diameters and various shell thickness, and some of them are presented in Table 1.
The advantages of the new core-shell particles relate to the Van Deemter function (Equation 13). The longitudinal diffusion coefficient *B* and the eddy dispersion term *A* are reduced by using the core-shell particles. The diminution of the *B* was presumed since a remarkable part of the column volume is occupied by nonporous silica. Thus, analytes cannot axially diffuse through it. The reduction of the eddy dispersion *A* was not expected. Decrease of the *A* comes from the tighter size distribution of core-shell compared to fully porous particles. Alternatively it comes from the reduction of the trans-column velocity biases that may result from the roughness of the external surface area of the core-shell particles.

Table 1. Particle structure and stationary phase chemistry of some commercially available shell packings.[12]

Vendor	Column/Product name	Average particle diameter (µm)	Shell thickness (µm)	Stationary phase chemistry
Advanced Material Technology	Halo	2.7	0.50	C18, C8, HILIC, RP-amide, phenylhexyl, pentafluorophenyl
Advanced Material Technology	Halo Peptide-ES 160 Å	2.7	0.50	C18
Agilent	Poroshell 300	5.0	0.25	C18, C8, C3
Agilent	Poroshell 300	2.7	0.50	EC-C18, SB-C18
Macherey-Nagel	Nucleoshell	2.7	0.50	RP-18, HILIC
Phenomenex	Kinetex	2.6	0.35	C18, XB-C18, C8, HILIC, pentafluorophenyl
Sigma-Aldrich	Ascentis Express	2.7	0.50	C18, C8, HILIC, RP-amide, phenylhexyl, pentafluorophenyl
Sigma-Aldrich	Ascentis Express Peptide-ES 160 Å	2.7	0.50	C18
Sunniest	SunShell	2.6	0.50	C18
Thermo Scientific	Accucore	2.6	0.50	C18, aQ, RP-MS, HILIC, phenylhexyl, pentafluorophenyl
Commercially not available	Eiroshell	1.7	0.35	C18
		1.7	0.25	
		1.7	0.15	

3.2.3 Monolithic Columns

Analysis time is possible to decrease using monolithic column by enhancing the flow velocity of mobile phase.[16] Monolithic columns consist of a single rod of porous material of silica or polymeric and they have bimodal structure which consists of mesopores and macropores. Size of these pores varies a little bit between first and second generation of silica-based monolithic columns. Silica-based monolithic columns have good permeability.[16,17] Raising the flow-through pore size and external porosity of the packing material leads to faster separation. The mass-transfer kinetics of analyte molecules is quicker through the monolith column than through the packed column of comparable same domain size and geometry.[10, 18] Also, the kinetic efficiency of monolith columns is comparable to packed with 3-4 µm porous particles.[10] Monolithic column material was first time developed by Hjerten *et al.*[19], Svec and Frechet[20] and Tanaka *et al.*[21] during the 1990s and the initial commercial versions (silica-based) was published in 2000 from Merck and Phenomenex (Chromolith™ and Onyx™, rescpectively). Various types of inorganic (for example silica, carbon and zirconia etc) and organic (poly(styrene-divinylbenzene), polymethacrylate and polyacrylamide etc) monoliths are possible to prepare, but solely silica-based, polymethacrylate and poly(styrene-divinylbenzene) are commercially available at the moment.[16] The organic monolithic columns are used typically in conventional HPLC but they are applicable for separation of macromolecules (e.g antibodies or proteins). However inorganic silica-based columns are more used in HPLC. Despite

the monolithic columns are considered as major innovation of column technology, very small number of LC separation applications are on a routine basis using by this column type. Reason for this is the lower performance in comparison to the other methods, for example core-shell technology and UHPLC.

3.2.4 High Temperature LC

One way to decrease the analysis time is to increase the temperature of the mobile phase ($60 < T < 200$ °C) in HPLC.[16] The higher temperature reduces the viscosity η of the mobile phase leading to higher diffusion coefficient for the compound, therefore increase the mass transfer and finally allows the use of high flow speeds. Temperature also reduce backpressure in the column with using of a constant flow speed. Resolution is not declined, however, due to column heating but pre-heating of mobile phase is useful to avoid the band broadening.[10] Horváth and Antia[22] expressed first that elevating temperature is a high effective way for large molecule separation. High temperature liquid chromatography (HTLC) suffers from limits like the small number of stable packing materials at high temperatures and furthermore the possible degradation of thermolabile analytes.[10, 12] In other words this procedure is not anyway relevant to pharmaceutical analysis because most of drug compounds are sensitive to heating and thus compound degradation is possible.

3.3 Method Transfer

Part of the conventional LC methods can be transferred to fast separation LC methods without losing sensitivity or resolution in pharmaceutical analysis.[10] When increasing sample throughput by transferring method, comparable method parameters must be used to sustain equivalent separations. To retain selectivity as changeless while transferring the method, operating conditions and column properties must be maintained consistent: the same or highly similar stationary phase chemistry must be used to maintain the selectivity. If the stationary phases are not equivalent, or just the two columns are not matched, disparities in selectivity can be distinguished. The alterations of the particle size and column length allow for rapid analytical methods by applying scaling factors. In that case during the analysis will not occur losing of sensitivity or resolution and this procedure is called geometrical transfer.

During method transfer adaptation of the injection volume to the column dimension is necessary to avoid an extra-column band broadening.[10,23] In that case sensitivity is maintained as equivalent level also and the similar separation factor is achieved using the adapted injection volume. It is determined as follows:

$$V_{i2} = V_{i1} \frac{d_{C2}^2 \cdot L_2}{d_{C1}^2 \cdot L_1} \quad (23)$$

where V_i is the injection volume, L is the column length and d_C the column diameter. The linear velocity of the mobile phase (v) should be kept constant (independent value of the column geometry) for a geometrical transfer. Thus, the new flow rate is computed as follows:

$$F_2 = F_1 \left(\frac{d_{C2}}{d_{C1}}\right)^2 \frac{d_{p1}}{d_{p2}} \quad (24)$$

where d_p is the particle size. In linear (or multilinear) gradient elution, the gradient profile can be decomposed as the combinations of gradient and isocratic segments. To generate same elution patterns, the gradient volume is scaled in proportion to the column volume. The ratio between isocratic step (and the equilibrating time) (t_{iso}) and dead time of column is adjusted using the following equation:

$$t_{iso2} = t_{iso1} \frac{F_1}{F_2} \frac{L_2}{L_1} \left(\frac{d_{C2}}{d_{C1}}\right)^2 \quad (25)$$

The initial and final gradient composition ($\%B$) has to be constant (slope segments). Thus the gradient time of transferred method is presented with the following way:

$$t_{g2} = \frac{\%B_{final1} - \%B_{final2}}{slope2} \quad (26)$$

Then the new scaled down (transferred) gradient times are obtained by exploring the equations:

$$t_{g2} = t_{g1} \frac{F_1}{F_2} \frac{L_2}{L_1} \left(\frac{d_{C2}}{d_{C1}}\right)^2 \quad (27)$$

The method can be transferred from customary LC method to faster LC method using the previous equations. In that case resolution and sensitivity are not suffering.

3.4 Applications of Method Transfer

Guillarme *et al.*[23] compared conventional LC method and fast and ultra-fast LC method in the pharmaceutical analysis. They also examined a successful method transfer from conventional LC method to fast and ultra-fast LC method in the gradient mode in the pharmaceutical analysis. They did two tests: a first approach was to improve the conventional HPLC method using short column packed with small particles. The second approach was performed by transfer method from HPLC to

UHPLC. Both the mobile phase was comprised a gradient mixture of 0.01 % (v/v) formic acid in acetonitrile (ACN) and 0.01 % (v/v) formic acid in water.

A first approach[23] was tested by applied for the separation of a drug substance and several related impurities (undisclosed structures). The solution contained the active substance at 100 ppm and eight by-products at 40 ppm. Separations of the mixture parts were carried out on conventional column XTerra RP_{18} (150 × 4.6 mm, 5 μm) and three short columns XTerra RP_{18} (lengths 20-50 mm) that were packed with 3.5 μm particles. HPLC instrumentation (Merck) was optimized. Particular attention was paid to the dwell volume, or gradient delay. The UV/Vis-detector was used. Under the test the analysis time was possible to decrease even by a factor of 10 using fast-LC method (short columns) compared to original HPLC method (conventional column). Loss in resolution and sensitivity was acceptable. The use of short columns is valid in the gradient mode for decreasing the time of analysis if the system is optimized (Equations 23-27).
A chromatogram with conventional HPLC system (Figure 6) and a chromatogram with optimized and fast HPLC system (Figure 7) are presented.

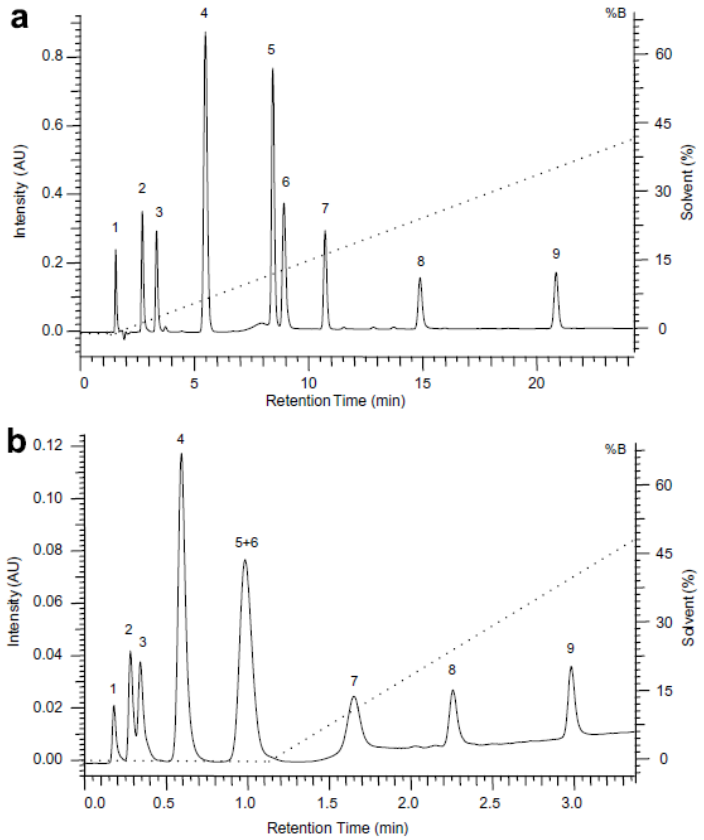

Figure 6. Separation of a mixture containing the API (5) and impurities in gradient mode with conventional HPLC system. **a**) Column XTerra RP_{18} 150 × 4.6 mm, 5 µm; flow rate: 1000 µl/min; injection volume: 20 µl; total gradient time 45 min. **b**) Column XTerra RP_{18} 20 × 4.6 mm, 3.5 µm; flow rate: 1430 µl/min; injection volume: 3 µl; total gradient time 4.2 min.[23]

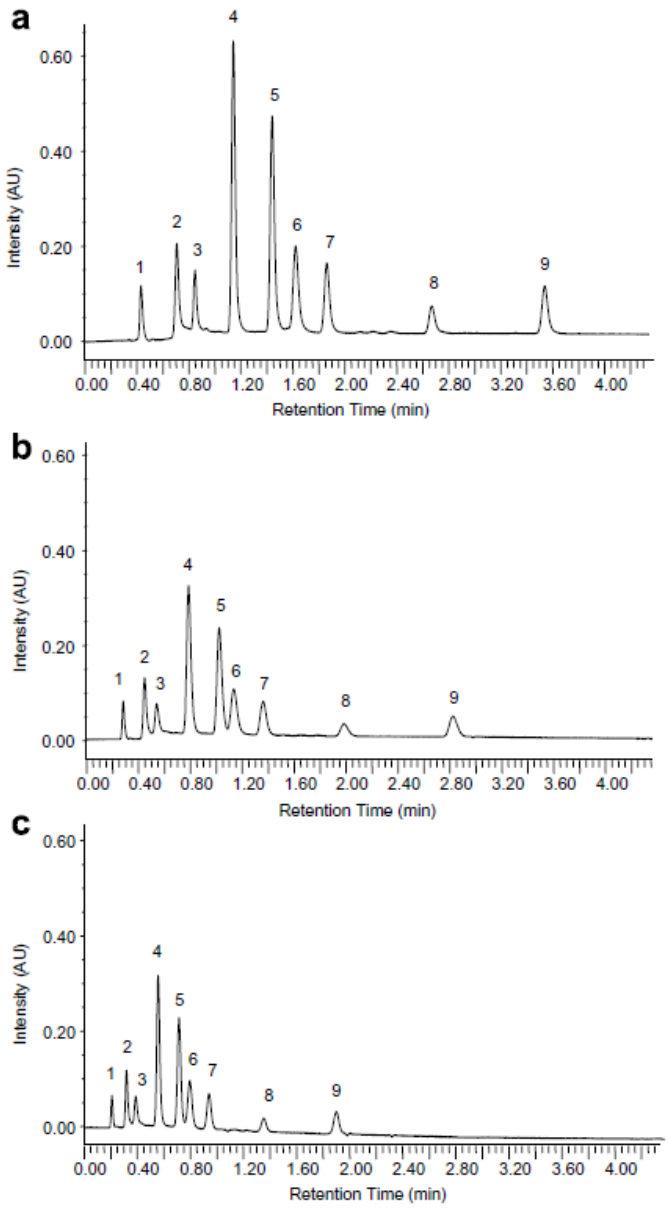

Figure 7. Separation of a mixture containing the API (5) and impurities in gradient mode with optimized HPLC system. **a**) Column XTerra RP_{18} 50 × 4.6 mm, 3.5 µm; flow rate: 1430 µl/min; injection volume 7 µl; total gradient time 10.5 min. **b**) Column XTerra RP_{18} 30 × 4.6 mm, 3.5 µm; flow rate 1430 µl/min; injection volume: 4 µl; total gradient time 6.3 min. **c**) Column XTerra RP_{18} 20 × 4.6 mm, 3.5 µm; flow rate 1430 µl/min; injection volume: 3 µl; total gradient time 4.2 min.[23]

The second tested procedure[23] was performed using UHPLC equipment (Waters Acquity UPLC System) with very high pressure (even 1000 bar) and a short column packed sub-2 µm particles. A complex pharmaceutical mixture contained active substance at 100 ppm and 11 by-products at 40 ppm was analyzed. The separation of these 12 compounds was originally performed using HPLC system and XBridge C_{18} column (150 × 4.6 mm, 5 µm). In this study the method transferred to

UHPLC using Acquity BEH C_{18} (50 × 2.1 mm, 1.7 µm) column. The UV/Vis-detector was used. A pharmaceutical mixture was separated allowing a diminution of the analysis time even by a factor of 15 versus the former conventional method without compromising the separation quality. Chromatographic performance, or resolution and peak capacity was reduced a little, however. Dwell volume should take into account when transferring method, because it signifies the most detrimental parameter for gradient separation.

Chromatograms with conventional HPLC system and UHPLC system are presented in Figure 8.

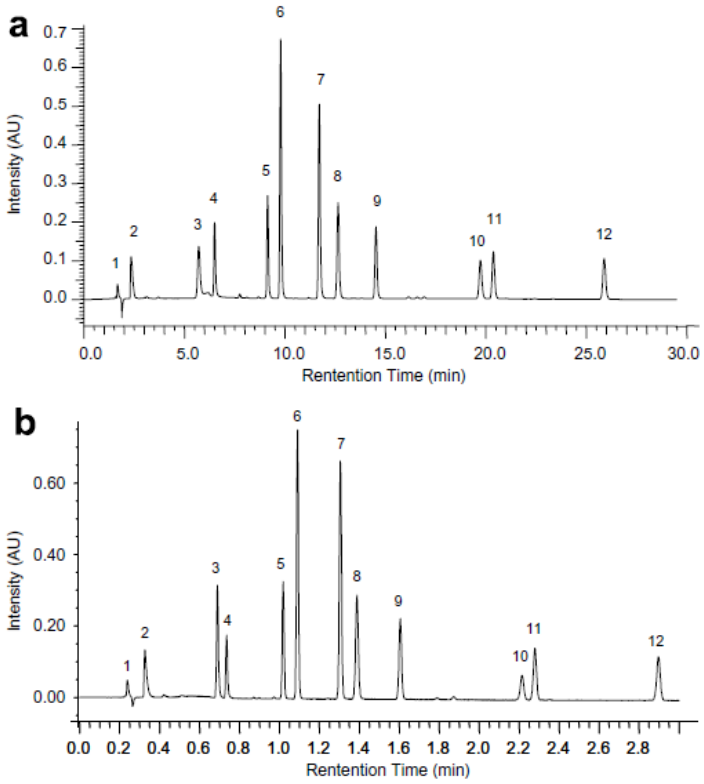

Figure 8. Separation of a mixture containing the API (6) and impurities in gradient mode with HPLC and UHPLC system. a) HPLC system, column XBridge C_{18} 150 × 4.6 mm, 5 µm; flow rate 1000 µl/min; injection volume 20 µl; total gradient time 45 min. b) UHPLC system, column Acquity BEH C_{18} 50 × 2.1 mm, 1.7 µm; flow rate 610 µl/min; injection volume 1.4 µl; total gradient time 5.1 min.[23]

The research[23] demonstrated that it is possible to transfer from slower analyses methods to the faster methods in some cases.

Guillarme *et al*. developed freely usable Excel program called "HPLC calculator" which is software for chromatographic performance evaluation and HPLC method transfer.[23-25] It calculates optimal conditions for method transfer using Equations 23-27 and the calculator is allowed for both gradient

and isocratic mode. It's available on the website of University of Geneva, Laboratory of analytical pharmaceutical chemistry.

4 Detectors

4.1 Common Detectors

An HPLC detector measures the concentration or mass of eluting analytes.[2] There are various type of detectors and their sensitivity varies. Ability to detect analytes varies also; some detectors can detect only specific compounds and the others can detect universal compounds.

Liquid chromatography with UV/Vis (ultraviolet/visible) absorbance detection is the most used technique that is applied for control of impurities in drug substances in the European Pharmacopoeia (Ph.Eur).[26,27] Therefore, UV/Vis detection is the most popular detection technique in HPLC analysis in the pharmaceutical industry and it is due to its broad linear range, quite low cost, high sensitivity, ease of operation and its compatibility with several mobile phase solvents, in both gradient and isocratic eluent modes.[28] This sort of detector monitors the absorbance of UV or visible lights by analytes in the eluent.[2] This is usable in the most cases because most drugs have chromophore group in the structure so they have absorbance in this wavelength range. There are a deuterium source and a monochromator in a typical UV/Vis detector. A monochromator is a movable grating which selects a wavelength through an exit slit and it focuses the light through a small flow cell. Two photodiodes measure light intensities of the sample and the reference beams. The principle for an absorbance detection can be presented as Beer-Lambert law as follows:

$$A = \varepsilon bc \qquad (28)$$

where A is the measured absorbance, ε is the wavelength dependent molar absorptivity, b is the path length and c is the analyte concentration. Transfer of electrons from $\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$ or $n \rightarrow \sigma^*$ molecular orbitals cause the most of UV absorption bands. Therefore, organic functional groups like carbonyl, amide, nitro and ketone groups and aromatic compounds can absorb in the UV region. In practice, presence of the analyte in the detector flow cell produces the change of the absorbance, and if the analyte absorbs greater than the background, or mobile phase, a positive signal is obtained.[4]

A graph of an UV/Vis detector is presented in Figure 9.

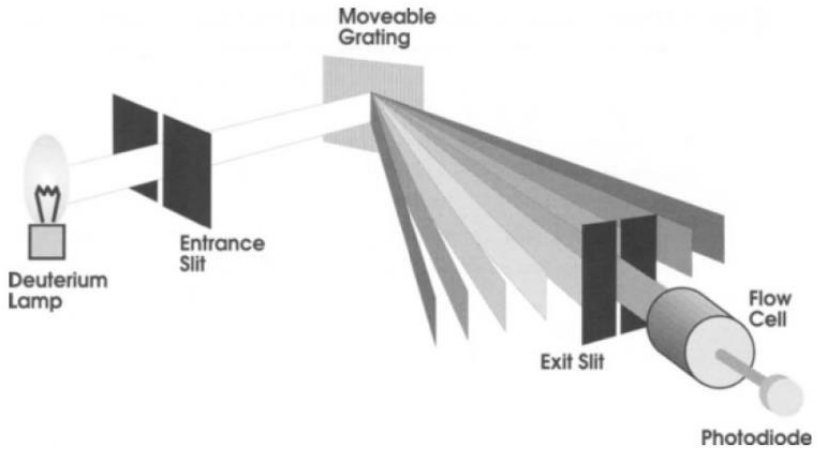

Figure 9. Schema of the UV-Vis detector.[2]

Another common detector used in pharmaceutical analyses is a photodiode array detector (PDA), or diode array detector (DAD).[2] Like UV detection, the response of PDA is for specific compounds. Operation of a PDA detector is quite similar than UV/Vis detector, it monitors the absorption by analytes in the HPLC eluent and provides UV spectra. Spectrum from the deuterium source passes through the flow cell and with the help of grating it disperses on to a diode array element. The diode array element measures the intensity of light at each wavelength. In other words, PDA monitors the UV absorbance over a wide range of wavelengths, while UV detector monitors the UV absorbance at a selected wavelength.[4] Nowadays the PDA have sensitivity performance equivalent to that of UV/Vis detector.[2]

A graph of a photodiode array detector is presented in Figure 10.

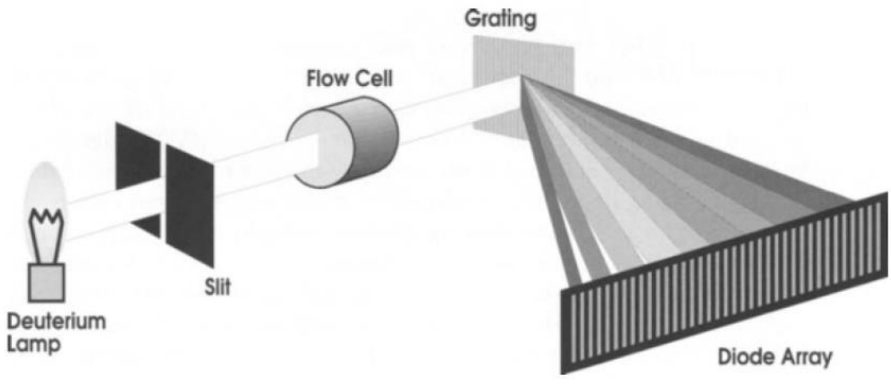

Figure 10. Schema of the PDA detector.[2]

Moreover, general alternatives to UV detection are refractive index (RI) and mass spectrometry (MS), but they are expensive and they have some lack of sensitivity.[29] RI detector measures the difference in refractive index between the sample cell containing the analyte in the eluent, and the

reference cell containing pure eluent.[2] RI detectors can detect the universal compounds (also compounds with low chromophoric activity) but RI detectors are incompatible with gradient elution.[29] There are also many less commonly employed detectors as chemiluminescence nitrogen detector, nuclear magnetic resonance (NMR), fluorescence detector, electrochemical detector (ECD) and conductivity detector.[2,29]

4.2 Aerosol-Based Detectors

As has already been mentioned, the pharmaceutical compound has to possess a UV-absorbing chromophore in order to be detected in HPLC analysis when UV detection is only used.[30] Anyway, some of active pharmaceutical ingredients (API) or some impurities do not very effective absorb UV radiation and they will not be detected in routine HPLC analysis. Many classes of compounds like amino acids, carbohydrates and polymers lack chromophoric groups. They have lack of π-electrons and they are hard to detect in the UV detection up to at low wavelengths. In that case, some other detection technique coupled to HPLC for non-UV-absorbing compounds must be used, and an option is aerosol-based detection. Aerosol-based detectors are for example evaporative light scattering detector (ELSD), charged aerosol detector (CAD) and condensation nucleation light-scattering detector (CNLSD).[29] The response caused by these aerosol-based detectors are independent of the chemical structure of the analyte compound.[29,30]
A diagram of main components and processes in aerosol-based detectors is presented in Figure 11.

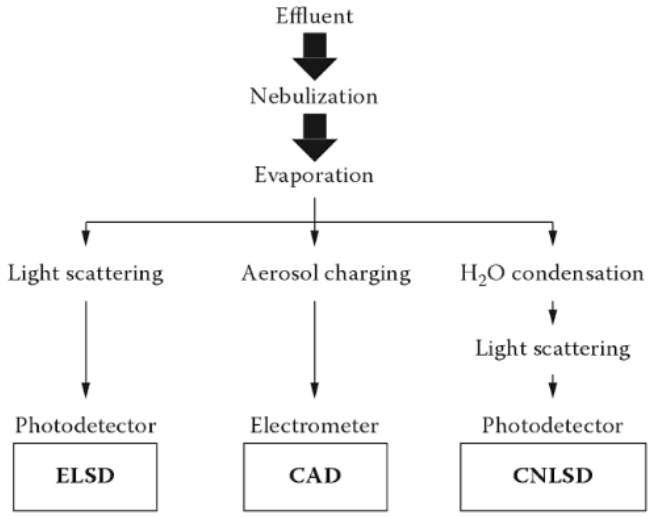

Figure 11. Graph of main components and processes in aerosol-based detectors ELSD, CAD and CNLSD.[29]

Good characteristics of aerosol-based detectors are relatively low cost, wide selection of commercially available models, resistance, low limit of detection (LOD), a response is independent of analyte's molecular structure and acceptable precision.[29] However, there are also limitations of these detectors, like requirement for volatile mobile phases, nonlinear response and sensitivity may suffer when analyzing volatile and semi-volatile compounds. ELSD and CAD are mass-dependent detectors, whereas the response of UV and CNLSD are concentration-dependent.[30]

This thesis focuses on the aerosol-based detectors based on the reason that these detectors are universal: the response is for any analyte, not only for a certain class of compounds. The detectors will be elaborated further and dealt with some applications in pharmaceutical analytics in the next sections of this chapter.

4.2.1 Evaporative Light Scattering Detector

Evaporative light scattering detector (ELSD) is one of the detectors that are used in conjunction with HPLC. ELSD was introduced in the 1960s by Ford and Kennard.[31]

4.2.1.1 Operation

There are three separation regions in the ELS detector and they are nebulization, desolvation and detection.[32] They function as follows: The solvent flows from a liquid chromatography to ELSD and the detection operates by nebulizing this flow and entraining the resulting droplets in a carrier gas stream. In that case the mobile phase is evaporated from the droplets. An analyte keeps as a "dry" solute particle in the gas flow and flows to the detector, and this is possible when the analyte is less volatile than the mobile phase. These analyte particles scatter the light beam in the ELSD and at the same time the amount of scattered light is measured. There is a relationship between scattered light and the concentration of material eluting. In the nebulization region the solvent is ran through the nebulizer and a fine aerosol is eddied out. In the process the solvent mixes with a carrier gas (usually N_2), and the result are droplets that form the aerosol. The formed aerosol streams to a drift tube (Figure 12). If the gas flow is high the produced droplets are small and then less heat is required to evaporate the solvent. It works also conversely, in other words if the gas flow is low the produced droplets are large and more heat is required to evaporate the solvent.

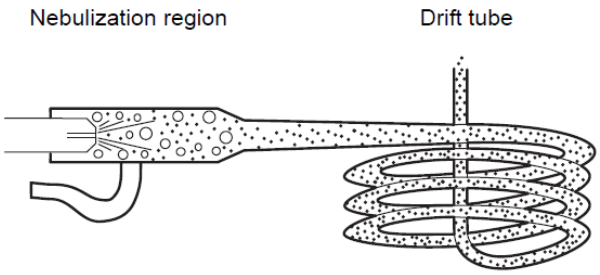

Figure 12. Nebulization region and drift tube in ELS detector.[32]

In the desolvation region happens evaporation of the mobile phase and dried solute particles stream in the drift tube.[32] The aerosol drops become smaller while they exiting the nebulizer and goes through the drift tube. Then the carrier gas sweeps the dried aerosol drops along to the detection region. It is important to use mobile phase that readily evaporate and desolvate. The best options are the solvents that have quite low viscosity and boiling point, for example methanol, ethanol, acetonitrile and water. It is not recommended to use viscous and high-boiling solvents because when using this type of solvents separation from the analyte molecules is not perhaps occurred properly. In that case the background noise is increased and the analyte signal response is decreased, which brings on the high LOD and low sensitivity (slope of the calibration curve). The evaporated solvents are condensed and captured in the solvent trap.

In the detection region, a light source impinges on the dried aerosol drops (analyte particles) and therefore the light is scattered and focused onto a photomultiplier tube (PMT) or a photodiode.[32] The intensity is measured in PMT. The size, or diameter of the analyte particles defines the scattering of the light. The intensity of the scattered light is measured at 60° relative to the excitation beam and this minimizes the light straying and polarization effects. Different sized particles express various angular distribution of the scattered light, and typically the larger particles cause more light scattering. The larger particles bring more intense signals and peak responses. PMT can convert the scattered light signal to a voltage, and it is recorded and analyzed. When scattering is stronger, the final signal is more intense on the ELS detection chromatogram. The scattered light is roughly proportional to the mass of material represented by a chromatographic peak. However the output of the ELSD has no direct link to the analyte molecular weight. Several factors can have also an effect to the mass response, for example specially the density of the analyte in a small dried aerosol particle.
The schema of the evaporative light scattering detector is presented in Figure 13.

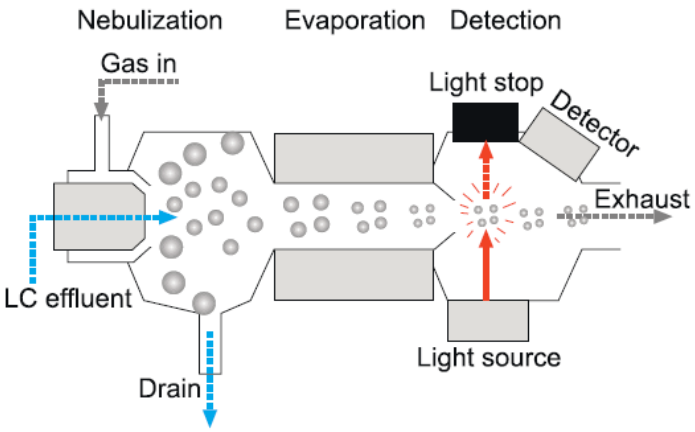

Figure 13. Schema of the evaporative light scattering detector ELSD.[33]

4.2.1.2 Principles of Light Scattering

Operation of both ELSD and condensation nucleation light-scattering detector CNLSD are based on light scattering and the phenomenon is described more accurately in this section.

There are three possible types of light scattering and they are called Rayleigh, Mie and Refraction-reflection.[32] The types are presented in Figure 14.

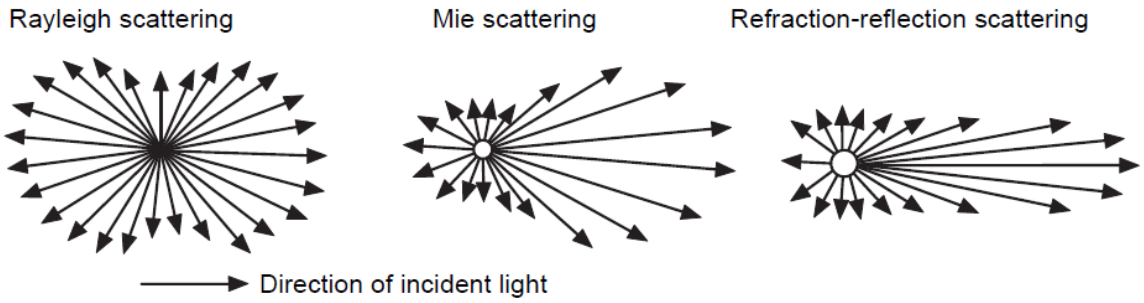

Figure 14. The types of light scattering.[32]

Resulting dry analyte particle comes from a nebulizer can presented as follows:

$$D = D_0(c/p)^{1/3} \qquad (29)$$

where D_0 is average liquid droplet diameter, c is concentration of the analyte and p is density of the dry analyte.[32] The type of light scattering depends on the size, or the diameter of the analyte particles that goes through the light beam, because the ratio of particle diameter to the incident wavelength $\frac{D}{\lambda}$ defines the type of light scattering.

If the analyte particles are very small, the Rayleigh scattering occurs where $\frac{D}{\lambda} < 0.1$. The scattered light is proportional to D^6 and thus scattered signal is proportional to c^2.[32]

In the event that the analyte particles are a little bit larger, the Mie scattering happens where $\frac{D}{\lambda} > 0.1$ but < 1.0. The scattered light is proportional to D^4 and hence the scattered signal is proportional to $c^{4/3}$.

Assuming that the analyte particles are large, the Refraction-reflection scattering occurs where $\frac{D}{\lambda} > 1.0$. The scattered light is proportional to D^2 and consequently the scattered signal is proportional to $c^{2/3}$.

The concentration of the analyte as a chromatographic peak represents changes, because concentration varies between near-zero at the baseline and maximum. This maximum corresponds to concentration of the injected sample and also retention time, injection volume and column efficiency. Then the concentration returns from the maximum to the near-zero. Assuming that the analyte concentration is high enough, the diameter of a dry particle can range all scattering regimes (Rayleigh, Mie and Refraction-reflection scattering).

4.2.1.3 Applications in Drugs Analytics

As said, some APIs or impurities do not very effective absorb UV radiation and they will not be detected in HPLC analysis with UV detection. In that case, some other detection like ELSD coupled to HPLC is an option for non-UV-absorbing compounds.[30]

Vervoort *et al.*[30] compared in their study the application of ELSD and charged aerosol detector, CAD in RP-HPLC. Also, they applied UV detection. Pharmaceutical compounds were APIs that have a UV-absorbing chromophore and thus they can be detected using UV detection, too. The research examined the various performance characteristics like sensitivity, accuracy, linearity and precision of both detector (ELSD and CAD) coupled to HPLC. Samples were Levamisole, Azaconazole, Liarozole, Flubendazole, Cinarizinne, Isoconazole, Sabeluzole, Domperidone, Ketoconazole and Itraconazole. A stock solution's concentration was 1.00 mg/ml of all compounds and serially diluted to the different concentrations using water/*N,N'*-dimethylformamide as the dilution solvent. Solute concentrations were 1.00, 0.75, 0.50, 0.25, 0.10, 0.075, 0.050, 0.025, 0.010, 0.0075, 0.005, 0.0025 and 0.001 mg/ml.

Chromatographic experiments were performed on a HPLC system (Waters) equipped with diode array detector DAD (Waters 2996) and ELSD detector (Waters 2440) or Corona CAD detector (ESA Analytical).[30] 10 mM ammonium acetate in 95:5 water:ACN as eluent A and acetonitrile as eluent B was used and all experiments was performed using 150 mm x 4.6 mm X-Bridge C18 column packed with 3.5 µm particles (Waters). Every injection volume was 6 µl and every experiment was repeated at least three times. Both aerosol-based detectors were able to detect all chosen compounds. Chromatograms of the concentration 0.10 mg/ml sample with both detectors are presented in Figure 15. Peak height of HPLC-CAD is in AU and peak height of HPLC-ELSD is in mV.

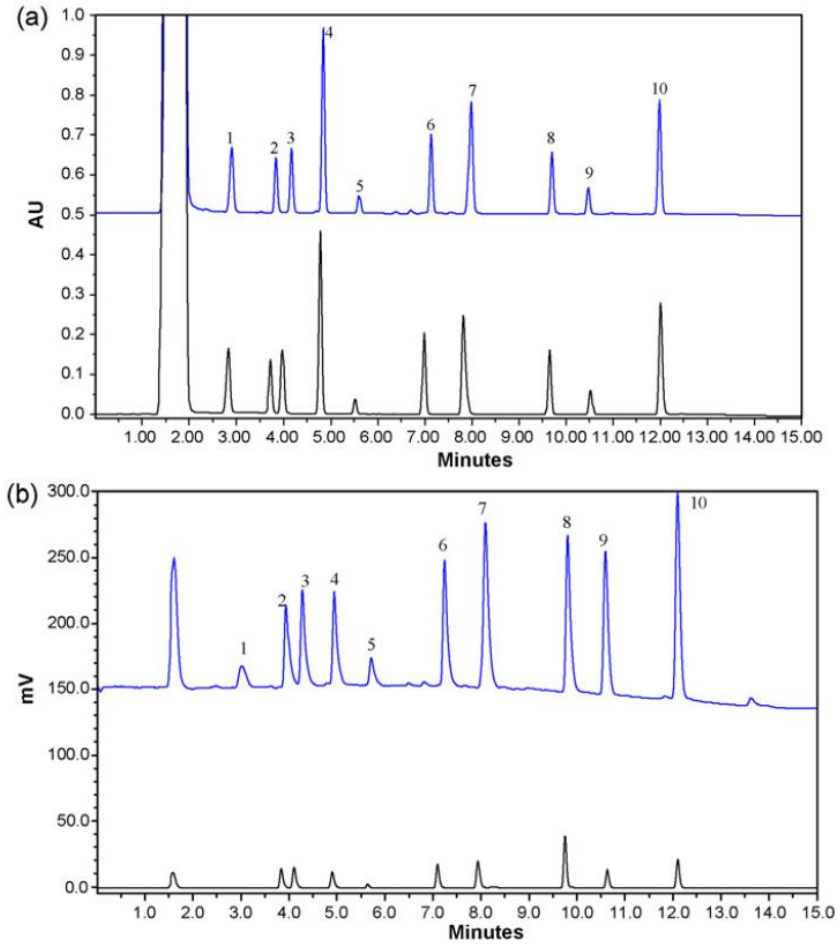

Figure 15. Chromatogram of the concentration 0.10 mg/ml sample a) UV (upper) and CAD (lower) signals of HPLC equipped with CAD and b) UV (upper) and ELSD (lower) signals of HPLC equipped with ELSD. Peak identification: 1. Levamisole, 2. Liarosole, 3. Domperidone, 4. Flubendazole, 5. Azaconazole, 6. Ketoconazole, 7. Isoconazole, 8. Itraconazole, 9. Sabeluzole, 10. Cinirazine.[30]

Peak number 5 or Azaconazole had a lower response than the others compounds. The response was especially lower using ELSD (Figure 15, b)) and according the study it came from thermal degradation because of drift tube temperature.[30]

The research[30] discovered that the CAD is more sensitive than the ELSD but both detectors were able to detect all pharmaceutical compounds studied with sufficient accuracy and sensitivity required in an analytical laboratory. Both detectors generated non-linear response curves which was possible to transformed into linear response curves by a log-log transformation. Then the linearity of CAD was better than the linearity of ELSD. However, this non-linear response behavior is one disadvantage of these detectors. Both CAD and ELSD had worse system repeatability than UV detection. Repeatability was estimated by evaluating RSD on the area response of repetitive injection of the same reference. Detection problems are increased if the compounds are sensitive to thermal degradation and if the ELSD with elevated drift tube temperature is used. CAD nebulization and drying of the effluent occur at room temperature thus thermal degradation does not happen.

In the other research Almeling and Holzgrabe[26] examined the use of ELSD for quality control of drug substances. They evaluated the influence of various LC and ELSD parameters and eluent parameters on the appearance of spike peaks. Additionally, opportunities to avoid occurrence of spike peaks in the chromatograms was research subject. The terms “spikes” and “spike peaks” in the context of the research was chosen to describe the phenomenon of non-reproducible peaks eluting after the principal peak in concentrated analyte solutions. According the study for a prospective use of the ELSD for the quality control (impurities control) in compounds of pharmaceutical use, requirement is that system has to bear high concentrations of APIs and moreover it is capable to detect impurities which can have very low concentrations. Therefore, it’s important to avoid spike peaks in the chromatograms because otherwise it is impossible to proper evaluate the impurity profile. The effect of various eluent and ELSD parameters (eluent composition and flow-rate, ELSD scavenger gas flow-rate and evaporation temperature) on the occurrence of spike peaks was studied. Moreover, during the study it was noticed that use of eluents containing low amounts of organic modifier is recommended because it decreased the appearance of spike peaks. The experiments were performed on a HPLC (Waters, 2695) equipped with ELSD (Polymer Laboratories, 2100). Tests were performed using LC instrument without analytical column or as flow injection analysis (FIA). A restriction coil was used to get system pressure. FIA was chosen because the goal of research was to study the dependence of the occurrence of spike

peaks on different eluent composition and flow, and ELSD settings. Samples were L-aspartic acid (Asp) (Sigma-Aldrich), Mexiletine HCl (ICN) and α-cyclodextrin (alfadex; Roquette).

First Almeling and Holzgrabe[26] tested the effect of eluent flow, eluent composition and ELSD settings as ELSD scavenger gas flow-rate and the nebulizer and evaporation, or drift tube temperature on the ELS detector response, or sensitivity. The ELSD response is determining for the evaluation of low concentration of impurities and thus the effect of the parameters was investigated using Mexiletine HCl and Asp test solutions. It was found found that the detector response (detector signal) increased when the mobile phase composition included a lot of organic modifier. The peak area decreased if the eluent flow-rate increased, and the detector signal decreased if the scavenger gas flow-rate increased. When the influence of the drift tube temperature was studied, it was noticed that the highest signal intensity was at temperature 60 ℃. Next, they investigated the effect of the eluent flow, eluent composition and settings on the appearance of spike peaks. It was noticed that spike peaks increased if the amount of organic modifier (MeOH or ACN) of the mobile phase increased. Spike peaks reduced if the eluent flow-rate increased and if the ELSD scavenger gas flow-rate was optimal. Moreover, spike peaks reduced if the temperature was 60 ℃ but did not disappear at higher evaporation temperatures.

According the research[26] it is possible to couple HPLC to an ELSD and it may be an applicable method to analyze the compounds that do not have UV-absorbing chromophore. The low limit of detection is a main factor when controlling impurities in pharmaceutical substances. Many parameters improve method sensitivity but at the same time they can cause the occurrence of spike peaks and the evaluation of the impurity profile becomes more difficult. It would be good to find the best compromise between them while the LC-ELSD method is developed for the control of impurities in pharmaceutical compounds.

4.2.1.4 Limitations

There are a few limitations when applying ELS detection.[32] It’s good to take into account for example that ELS detector should be the last detector in a series, because it destroys the analyte by causing the particle scattering. The possibility is also that the ELS detector is placed upstream of others and the column effluent is split to the both detectors from the LC thus the detector receives own flow from the liquid chromatography. Some problems are related to ELS detection when it is used for assays, because it is missing linearity over wide concentration range. In the other words,

the response is not linear. It is recommended to experiment with a variety of best fits using for instance linear responses for the compounds that will be analyzed, and moreover establish groupings for predictable concentration ranges. The detector has a tendency to respond equally to all particulates, so possibly any particle can interfere the signal of analyte, for example particulates in low-grade solvent. In this case, troubled background noise can occur (lack of selectivity). ELS detector's sensitivity to the any particulates raises noise and differences in the quality of mobile phases cause an increase in signal-to-noise variation for a certain method. Possibly also stationary phase components can disengage from the column and these particulates can join to the analyte flow. The number of unwanted particulates can be decreased by filtering liquid chromatography effluent and the equipment's carrier gas. Volatility of analyte compounds cannot be same than the mobile phase has because in that case ELS detection does not working properly. If they have identical volatility, it is not possible to evaporate only the mobile phase from droplets without evaporating the analyte also at the same time. Then it is impossible to detect analyte compounds. The ELS detector is sometimes a little sensitive to baseline drift, which is due to gradient changes in a liquid chromatography separation. Detector's performance is often dependent on the effect of changing solvent composition, which influences the ability of the nebulizer to form droplets and impact their size.

4.2.2 Condensation Nucleation Light Scattering Detector

Allen and Korophack[34] introduced the condensation nucleation light scattering detector (CNLSD) in the 1990s. It is improved version of ELSD, because CNLSD can overcame the particle size limitations to light scattering detection for liquid chromatography, in which case the sensitivity improves.[29] The novel technique bases on the condensation nucleation. Later the first commercial application of CNLSD was published in the end of 2000s and it is known as the nano quantity analyte detector (NQAD).[35]

4.2.2.1 Operation

Also, CNLSD works by utilizing the process of aerosol production like the other aerosol-based detectors.[33,34] Typically, pneumatic nebulizer produces the aerosol of the analyte and the mobile phase components and then this aerosol is dried (desolvation) to produce the aerosol of involatile analyte. After that the aerosol transfers through a filter (diffusion screens) and the particle size

distribution is modified. Then the intermediate step of condensation nucleation, or CN, is started. Supersaturated vapor n-butanol is condensed onto the dry analyte particles, if the CNLSD at the research stage is in question. If the commercially available NQAD is in question, water is condensed onto the dry analyte particles. This increases the size of the particles and then they can be detected by direct light scattering. Therefore, they are converted into droplets that are detectable. The size of particles is grown because the particles are too small to detect. Consequently, CNLSD overcomes the particle size limitations to light scattering detection. With condensation nucleation particles are grown to more effective at scattering light by first saturating the gas surrounding the particles with the vapors of a condensable fluid and next passing the mixture through a condenser. Then very small particles ($<$10 nm), have been grown to at least $>$10 nm. The mass of the particles increases during the condensation nucleation process and light scattering intensity is approximately dependent on the particle mass, and so the signal is increased. In the case of n-butanol, supersaturation and condensation nucleation of the vapor is caused by a heated saturator and a cooled condenser and then thermal diffusivity of the carrier gas is greater than the mass diffusivity of n-butanol. In the case of water, the condenser is held at a higher temperature than the saturator and then mass diffusivity of water is greater than the thermal diffusivity of the carrier gas.

As described by Equation (29), the size of the dry particles (after evaporation of the volatile eluent) is related to concentration of non-volatile analyte.[33,34] Diameters of the dry particles will be from around 10 nm to around hundreds of nanometers for higher concentrations. Diameter will be about 10 nm if typical HPLC-CNLSD concentration LOD would be approached. The response of the CNLSD detector is dependent on the concentration. The process of the condensation nucleation can be defined as a detection efficiency curve, where the efficiency of particle growth and detection vs. particle size is plotted as a function of particle diameter. The diameter of the smallest particle which size can be increased by condensation nucleation and which can be detected, can be estimated as the size-threshold for detection. All particles above this certain threshold size will be grown to approx. the same size during the condensation nucleation.
The schema of the condensation nucleation light scattering detector is presented in the Figure 16.

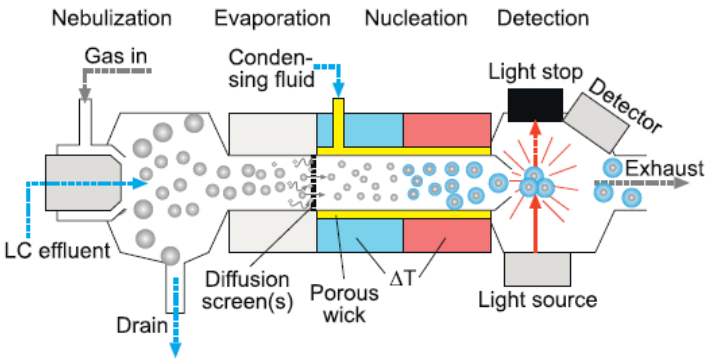

Figure 16. Condensation nucleation light scattering detector CNLSD/nano quantity analyte detector NQAD.[33]

4.2.2.2 Applications in Drugs Analytics

Many researches and applications of condensation nucleation light scattering detection or nano quantity analyte detection have been published in the field of pharmaceutical analysis.[33] Molecules are both bio/large and small molecules. Many of the studies are comparative of CNLSD/NQAD to CAD, ELSD or UV. According the results of several studies NQAD was much more sensitive than ELSD, but sensitivity between NQAD and CAD or UV varies. Magnusson *et al*.[33] presented on the review article, that discusses aerosol-based detectors for liquid chromatography, a few pharmaceutical analyses based on CNLSD/NQAD.

For example, one of these studies is a research where Hutchinson *et al*.[36] compared four aerosol-based detectors: NQAD, two different CAD detectors (Corona CAD and Corona Ultra) and ELSD. They evaluated the sensitivity and performance of each detector under a variety of chromatographic conditions. Samples were pharmaceutically relevant compounds, small molecule analytes that are commercially available: quinine HCl, benzyltrimethylammonium chloride, dibucaine HCl, labetalol HCl, amitriptyline HCl, sucralose, ibuprofen, aminobenzophenone, triphenylmethanol, linoneic acid and (±)-alpha-tocopherol. They were separated on a RP-UHPLC system. These separations (gradient mode) were compared on all aerosol-based detectors and further with UV detection, and only analytes containing chromophores could be detected using UV detector while aerosol-based detection was applicable to all analytes. The study discovered that NQAD expressed the highest sensitivity of concentration 10 ng/ml. The equivalent values were 76 ng/ml for the Corona CAD, 250 ng/ml for the Corona Ultra and 490 ng/ml for ELSD. The best precision was for the Corona detectors. However, NQAD and ELSD exhibited less peak dispersion. For instance a critical pair, dibucaine and amitriptyline, was separated only when NQAD and ELSD were used.

Another interesting study is by Olšovská *et al.*[35] where they developed the reversed phase isocratic UHPLC-NQAD technique for determination of compounds with low UV absorption. These compounds were macrocyclic antibiotic compounds or macrolides: erythromycin (ERY), clarithromycin (CLA), oleandomycin (OLE), troleandomycin (TRO) and roxithromycin (ROX). They lack chromophores and UV detectors are not applicable to detect them. The researchers compared the NQAD response to that of UV for these macrolide compounds and discovered that the NQAD was three times more sensitive than UV. The LODs were 3.0 - 5.4 µg/ml (15 – 27 ng) for NQAD, and 9.6 – 18.2 µg/ml (48 – 91 ng) for UV. Detectors were linear in the range of 3.1 – 100 µg/ml. Chromatographic conditions were the following for both detectors: column Waters BEH C18 (50 × 2.1 mm, 1.7 µm), column temperature 50 ℃, injection volume 5 µl, flow rate 0.4 ml/min, mobile phases water (A) and ACN (B), both containing 0.01 % or 0.04 % NH_4OH for NQAD and UV detection, respectively. Isocratic mode was used (A:B 55:45 v/v).

Chromatograms of separation of macrolides with NQAD and with UV are presented in Figure 17 and Figure 18. Difference between them is significant: NQAD detection is exceedingly more sensitive than UV detection for the macrolides.

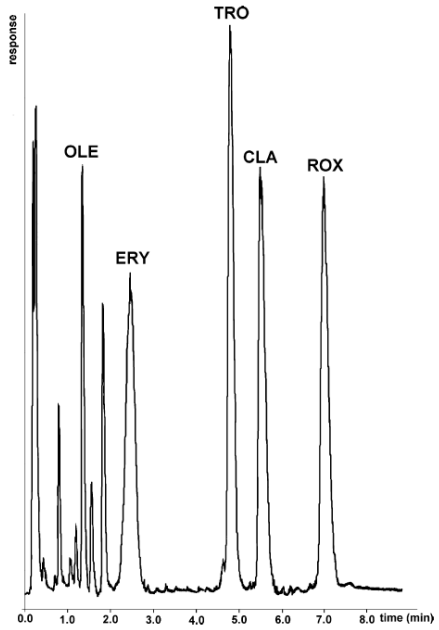

Figure 17. Isocratic separation of macrolides with NQAD detection. All macrolides at a concentration of 50 µg/ml.[35]

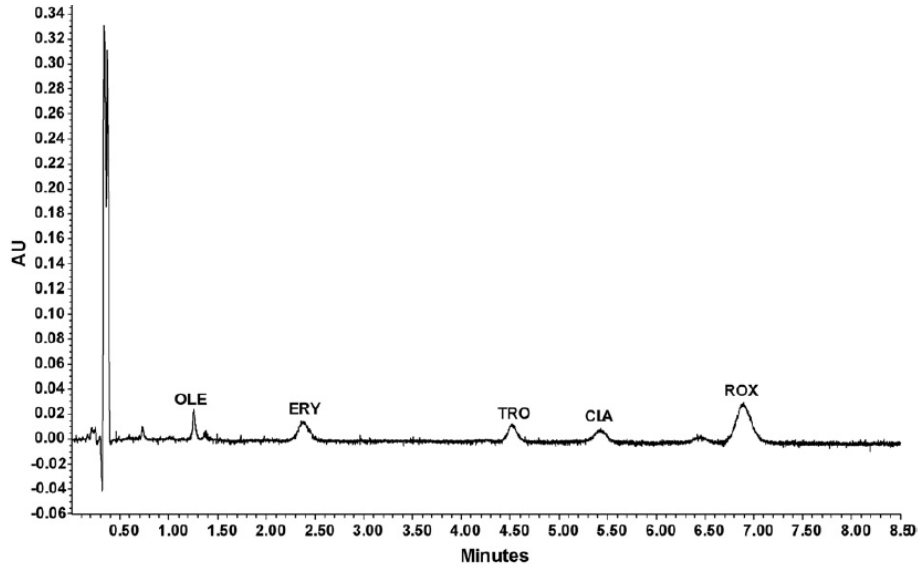

Figure 18. Isocratic separation of macrolides with UV detection. All macrolides at a concentration of 50 µg/ml, wavelength 194 nm.[35]

Moreover, in the research[35] a gradient separation of two of the macrolides and also ten additional antibiotic compounds was studied.[33] Researchers reached a conclusion that the NQAD was more sensitive than UV detection for some analytes: clindamycin, lincomycin, roxithromycin and clarithromycin. On the other hand, NQAD was less sensitive than UV for vancomycin, metronidazole and streptovitacin A. Sensitivity was equivalent for the others compounds.

4.2.2.3 Limitations

There are some limitations in relation to particle composition and condenser fluid on condensation nucleation.[33] Water is a typical condenser fluid in the NQAD and water appears to be more efficient for detection of hydrophilic particles than hydrophobic ones. However, detection of hydrophobic particles can be increased by adding a small amount of hydrophilic substance, for example sucrose. n-butanol condensation is less reliant on particle composition. The effect of the condensing fluid chemical characteristics coupled with analyte characteristics can partly expound analyte-to-analyte differences in observed response with HPLC coupled to CNLSD.

4.2.3 Charged Aerosol Detector

Charged aerosol detector (CAD) is quite new aerosol-based detection method for HPLC, and it was introduced in the 2000s by Dixon and Peterson.[37]

4.2.3.1 Operation

CAD departs from the others aerosol-based detectors, because its operation doesn't base on light scattering, but aerosol charging.[29] This aerosol charging can be effectively applied to small particles, which diameter is < 100 nm and it produces improved detection limits (LOD) and it is more sensitive compared to ELSD. Maximum sensitivity per particle mass S_m can be presented with the following equations:

$$\text{for } D < 10 \text{ nm} \qquad S_m = \frac{4.4\times10^5}{\rho} D^{3.6} \qquad (30)$$

$$\text{for } D > 10 \text{ nm} \qquad S_m = \frac{3.01\times10^{11}}{\rho} D^{-1.89} \qquad (31)$$

Equation (30) is for particles, which diameter is under 10 nm and equation (31) for particles, which diameter is over 10 nm.

Nebulization and evaporation are required also for the charged aerosol detector.[29,37,38] After HPLC eluent have passed the separation column, it is transferred to the CAD. There dried and purified nitrogen gas, or carrier gas is ionized by a high-voltage corona discharge needle and thus it becomes positively charged. Also in the CAD liquid phase transforms into small droplets while a carrier gas flows coaxially to the mobile phase. The charged nitrogen gas mixes with the aerosol particles and a charge transfers to them. Then particles go through the heated drift tube. In the drift tube the eluent is evaporated and the non-volatile material is formed. Then an electrical aerosol analyzer (EAA) detects the charged particles. The EAA is commercial available equipment for sizing particles and it was presented in the 1970s by Liu and Pui.[39] The electrical aerosol analyzer charges the particles when they pass a region of positive corona discharge. Next the fast-moving small particles are removed to a rod of negative charge. Then an electrometer of CAD measures the electric charge of the aerosol particles. The charge of the aerosol particle is proportional to its size (mass) and further proportional to concentration.

The schema of the CAD is presented in the Figure 19.

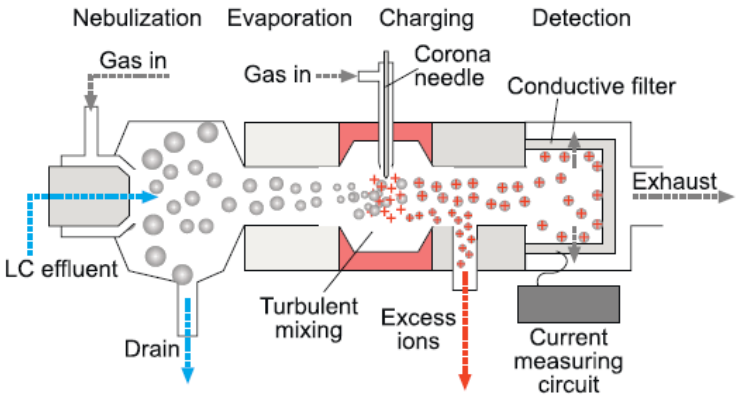

Figure 19. Schema of the charged aerosol detector CAD.[33]

4.2.3.2 Applications in Drugs Analytics

Such as ELDS, charged aerosol detector (CAD) can be used in the pharmaceutical industry for detection of compounds or impurities that are lacking UV-absorbing chromophores.[30] A condition for the utilization of that detector is the use of volatile mobile phases.[38] The CAD have sufficient linearity over a limited range of concentration and high sensitivity with LOQ down to 1 ng of substance on a column. Also, the CAD is easy to use and thus it is very applicable for both method development and routine analysis.

When HPLC is coupled with CAD, it is possible to use more organic modifier in the mobile phase compared for example to HPLC with UV detection.[38] For instance, while operated with HPLC-UV acetone is not normally used because it has a UV cut-off of 330 nm. However, this is not a problem when working with HPLC-CAD. Also, one of the advantages that the response of the CAD does not depend on particular functional groups. This is a benefit in cases where structural information of impurities is not available or where the detector response of impurities is not known. By taking this into account the CAD can used for the estimation of the UV relative response factors (RRF) of impurities that are unknown amounts in a mixture with API. Also, it is possible to compare the CAD and UV detector responses of the impurity and the API, and the RRF of the impurity with UV detection can be estimated. The charged aerosol detector response depends on the amount of the organic modifier in the mobile phase, and the detector signal can increase significantly if the mobile phase contains a lot of organic modifier. Compared with ELSD, CAD is generally more sensitive technique or it has higher detector response.

Almeling *et al.*[38] published a review that discusses the applications for CAD in the analyses of pharmaceutical industry. In the recent years, a wide variety of papers have been published demonstrating the potential use of HPLC-CAD in the field of pharmaceutical analysis. The review covers several successful studies of impurities control as a sample like Paclitaxel[40] (chemotherapy medication) where RRFs of impurities were determined by HPLC-CAD and compared to those acquired by HPLC-UV, or neuromuscular blocking drugs[41] where was developed a rapid and sensitive HPLC-CAD method to determine simultaneously drug substances and their degradation products or impurities in the pharmaceutical preparations. Moreover, the review explored assay determinations, for example, a study about statin drugs[42] where was developed and validated HPLC-CAD method for the assay determination of three different APIs (statins) in tablets. Moreover, they presented some further applications in pharmaceutical areas like the successfully application of HPLC-CAD in the pharmaceutical cleaning validation.[43] This application of cleaning validation is presented most accurately in the Chapter 5, Method Validation.

Shaodong *et al.*[28] studied operation of HPLC-CAD in assay determination as pharmaceutical analysis. They compared CAD with UV detection and ELSD methods for liquid chromatographic determination of anti-diabetic medications glipizide, gliclazide, glibenclamide and glimepiride. The detection methods were applied to the analysis of the anti-diabetic drug standards, real commercially available pharmaceutical tabs and anti-diabetic dietary supplements. The results were compared with reference to accuracy, precision, linearity and LOD. The standard stock solutions were prepared by dissolving the APIs in methanol and they were further diluted by MeOH to get five different concentrations 10, 30, 50, 70 and 90 µg/ml. Drug solution was made by dissolving drug power in methanol to acquire sample 50 µg/ml of the active component and the dietary supplement solution was made by extracting supplement power in methanol by sonication to obtain sample concentration of 2 mg/ml. The Series 200 HPLC (PerkinElmer) was used in all experiments. The used detectors were UV (PerkinElmer UV/Vis), ELSD 200 (Alltech Associates) and corona CAD Plus (ESA). All experiments were performed on a column GraceSmart RP-18 (250 mm × 4.6 mm, 5 µm) with water and ACN as the mobile phase and under the same chromatographic conditions for each detection method. Organic acid, 0.1 % formic acid, were added into the eluent and according the results it enhanced sensitivity and separation.

In the study[28] both UV detection and ELSD high coefficient of determination ($R^2 > 0.99$) for linear regression curve was achieved. In the case of CAD, a log-log transformation for the calibration curve was needed, and after this transformation a linear curve was achieved. The accuracies of all detectors were acceptable, expect the ELSD had a single poor result. The CAD showed the best

results of the recovery. The precision was expressed calculating the relative standard deviation (RSD) and according to the results both UV and CAD were significantly more precise than ELSD. The RSD of CAD was a bit worse than that of UV at low concentrations, but at higher concentrations the precision between CAD and UV was comparable. In the research, CAD expressed the best sensitivity because the most of the LOD values of CAD were best. CAD was somewhat better than UV detection but CAD was significantly better than ELSD.

4.2.3.3 Limitations

CAD has the same limitations than ELSD, for example the composition of the mobile phase modifies the response.[28] On the other hand, in the case a gradient elution is applied the peak areas possibly increase if the mobile phase contains a lot organic modifier. Like for all evaporation based detectors, it's important to notice that the solute should be less volatile than the mobile phase.[38] Also, a column that shows no bleeding should be selected because all non-volatile particles will be detected. Linearity of range is limited; the response of CAD is not directly linear over a broad concentration range. However, CAD is practical for most of assay determination or for the determination of impurities. In that case a standard in an appropriate concentration should be available. There is the limited suitability for gradient methods in the CAD due to strong dependence of the detector response on the mobile phase composition (organic modifier). In these instances use of a single external standard for the quantification of various analytes in a mixture is not possible. This problem can be solved using post-column compensation of the gradient by a corresponding counter gradient and corresponding dual-pump HPLC system solution.

5 Method Validation

5.1 General about Validation

The aim of the analytical method validation is to ensure that a developed analytical method is specific, reproducible, accurate and robust over the specified range that an analyte will be analyzed.[44,45] With the help of validation reliable working of the analysis during normal use can be justified. Also, it can be considered as documentation that the analytical method is capable for the intended use. Analytical procedures are used constantly during pharmaceutical development and

manufacturing pharmaceutical substances and products. Many important decisions are based on analytical results: the re-test time from the stability studies and the analytical control of the manufacturing process including release testing of the intermediates, to ensure the quality of the drug substance (for example the need for necessary measures if new impurities appear or if known impurities exceed the accepted levels, batch release or rejection and reworking of batches in the pharmaceutical industry). Thus, an appropriate performance of the analytical procedures is outstandingly significant in the process of making the accurate decisions and evading additional work. The laboratories in the pharmaceutical companies, or regulated laboratories must perform method validation according to the regulations that International Conference on Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), US Food and Drug Administration (FDA) and European Medicinal Agency[46] (EMEA) has established. The guidelines for pharmaceutical HPLC methods validation are available for example from FDA[47], US Pharmacopeia[48] (USP) and ICH[49].

Method development and method validation are strongly tied together.[44,45] Before validation a method validation plan need to be written, where is explained for example how the method will be used, what is the objective of the method and what level of characteristics including accuracy, sensitivity and LOD will be required. Establishment of the validation plan is followed according to validation standard operating procedure (SOP) and ICH quidelines, and it is written in a step-by-step instructions format. In analytical test method validation, it is recommended to perform a progressive validation, or pre-validation in which a basic data, for instance precision and robustness (stability of analytical solutions) are estimated. During method development the system suitability parameters (critical performance parameters) should be evaluated. These are for example capacity factor, repeatability, resolution, tailing factor and theoretical plates. Also, analytical equipment qualification is assessed. These preparative measures save time in the validation procedure.

5.2 Validation Characteristics

The requirements for analytical method validation by ICH and USP can be harmonized in the Table 2.[47-49] It comprises analytical method requirements for each method type: identification test, tests for impurities (quantitative and limit test) and assay tests. In addition to the requirements in Table 2, FDA validation requirements are sensitivity, recovery, reproducibility, robustness (also ICH), sample solution stability and system suitability.

Table 2. Requirement list for analytical validation[49]

Characteristics	Type of analytical procedure			
	Identification	Testing for impurities		Assay
		Quantitative	Limit test	
Accuracy	-	+	-	+
Precision				
Repeatability		+	-	+
Intermediate precision	-	+[1]	-	+[1]
Specificity[2]	+	+	+	+
Limit of detection	-	-[3]	+	-
Limit of quantification	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

+ = This characteristic is normally evaluated.

- = This characteristic is not normally evaluated.

(1) In cases where reproducibility has been performed, intermediate precision is not needed.

(2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

(3) May be needed in some cases.

Method validation is part of an integrated concept to ensure the quality, safety and efficacy of the pharmaceuticals.[45] Based on the validation characteristics and the requirements of the guidelines by competent authorities ICH, FDA and USP, each analytical procedure is validated with respect to parameters which are relevant to its performance.

Analytical validation characteristics for the liquid chromatographic methods are presented generally in the next sections of this chapter.

5.2.1 Accuracy

Accuracy is the measure of how close the test result obtained by the analytical method is to the true value. It can be determined in four ways depending on the type of analytical procedure.[44] The first approach is to estimate the accuracy by analyzing a sample of known concentration, or reference material and comparing the measured result to the true value. The second, accuracy can be assessed by comparing test results from the recently developed or validated method with results obtained by an existing alternative and accurate method. The third way to establish accuracy can be performed by spiking analyte in blank and it is based on the recovery of known amounts of analyte. For impurity analytical methods, three replicates of spiked samples are prepared at three levels over a

range that covers the specified impurity level of the sample. For assay determination, three replicates of spiked samples are prepared at three levels over a range of 80-120 % of the target concentration. The spiked samples are analyzed using the same analytical method and the same way as will be used in the final method procedure, and then the percent recovery is calculated. The fourth way is used if a blank sample is not possible to prepare without the presence of the analyte. In this case a standard is added, and this approach can also be used to determine recovery of spiked analyte.

5.2.2 Precision

Precision of an analytical method is a degree of repeatability and it is expressed as the percent relative standard deviation (RSD). Precision is performed at three different levels that are repeatability, intermediate precision and reproducibility.[44] Repeatability is expressed by performing tests in a short period of time under the same conditions for three replicates at three levels or by 6 replicates at one level. Intermediate precision is displayed by conducting analyses so that day, analyst or equipment etc. is different (within-lab), and possible effects of the individual variables should be monitored. Reproducibility is determined by testing samples in different laboratories and thus it is interlaboratory crossover research. The standard deviation (SD), relative standard deviation (RSD), coefficient of variation and the confidence interval are documented while precision is evaluated.

5.2.3 Specificity

Specificity is the capability of the chromatographic method to enough accurately measure the response of the analyte in the presence of all sample components in mixture.[44] Sample components may be for example impurities, degradation products, excipients or synthesis intermediates. The analyte response in mixtures is compared with the response of the only analyte-bearing solution. The analyte peak is evaluated for peak purity and the resolution is evaluated from the adjacent eluting peak. The possible alternative column is recommended to expressed during the method development. When acceptable resolution is obtained for the analyte and the mixture, the chromatographic parameters (flow rate, mobile phase composition etc.) are explored.

5.2.4 Limit of Detection

The limit of detection (LOD) denotes the lowest concentration of an analyte in a sample that is possible to detect.[44] In that case the analytical method is a limit test that determines whether or not an analyte is below or above a specific value, that is considered a limit value (concentration). Usually LOD is presented as signal-to-noise ratio (S/N), 3:1. There is also the other approach to define LOD for liquid chromatographic method and it is a mean of calculating detection limit as follows:

$$LOD = 3.3\times\left(\frac{\sigma}{S}\right) \qquad (32)$$

where σ is the standard deviation (SD) of the response and S is the slope of calibration curve. The standard deviation of the response can be defined based on the SD of y-intercepts or on the residual SD of the regression line, or on the SD of the blank. While developing the method and the LOD the documentation should perform properly, and a proper number of samples should be analyzed at the limit value to validate it.

5.2.5 Limit of Quantification

The limit of quantification (LOQ) denotes the lowest concentration of an analyte in a sample that is possible to define with acceptable precision and accuracy under the certain operational conditions of the analytical method.[44] LOQ can be determined as a signal-to noise ratio (S/N) of 10:1, but it is recommended to consider that quantification limit is a compromise between the required accuracy and precision and the concentration: if the concentration decreased then the precision increases. Like the LOD, also the LOQ for LC method can defined as a mean of calculating quantification limit as follows:

$$LOQ = 10\times\left(\frac{\sigma}{S}\right) \qquad (33)$$

where again σ is SD of the response and S is the slope of calibration curve. The σ again can be defined based on the SD of y-intercepts or on the residual SD of the regression line, or on the SD of the blank. While developing the method and the LOQ the documentation should perform properly, and a proper number of samples should be analyzed at the quantification limit value to validate it.

5.2.6 Linearity

Linearity means the ability of the method to achieve results that are proportional to concentration of the analyte within a certain range.[44] This range denotes a spread between the lower and upper levels of analyte and the concentration of analyte in the range is possible to determine with accuracy, precision and linearity using the intended method. While developing the method, the linearity should be determined using a minimum of five concentration levels including specific minimum specified range and it should consider the appropriately for the prospective analytical method. Acceptability of linearity is evaluated by exploring the correlation coefficient and *y*-intercept of the regression curve (the response v. concentration plot) and commonly the correlation coefficient >0.999 is accepted. Moreover, the requirement of the y-intercept is that it should be less than a few percent of the response got for the analyte (target level). One option to evaluate the linearity to the regression line is based on the residual sum of squares.

5.2.7 Robustness

The robustness of a method means its ability to stain stable by small variations in method parameters for example different HPLC columns or its temperature, flow-rate, percent organic solvent or pH of buffer in the mobile phase.[44] Robustness is considered at early stage, in the method development. If a method is sensitive to variations in method parameters and thus the results are varied, the parameters should be appropriately controlled and documented during the method development.

5.3 HPLC-CAD for Pharmaceutical Cleaning Validation

Cleaning of the manufacturing equipment is a critical step in the pharmaceutical manufacturing process.[38,43] Therefore, it is one of the major analytical application in the pharmaceutical industry. Guides of good manufacturing practise[50,51] (GMP) require that the equipment involved in pharmaceutical manufacturing has to be cleaned complied with specific low-level specifications (assay, LOD etc.). FDA requires that pharmaceutical companies validates their equipment cleaning method and the typical limit of detection is at least 10 ppm of the analyte. Cleaning method validation universally contains a separation of the pharmaceutical substance by HPLC and quantitation of the residual material on the manufacturing equipment based on a reference standard.

Analytical methods for the cleaning validation are commonly alike to the impurities control methods or assay determination methods, and HPLC equipped with UV/Vis detection is the most common technique for this purpose. Some residual materials, APIs and excipients do not have a chromophore in their structure, thus they have low UV response. In this case it will be problematic to detect them using the common technique.

Snow and Forsatz[43] examined how HPLC-CAD can successfully applied in addition to HPLC-UV detection for cleaning validation. In the study, several traditional wash solvents like water, methanol, ethanol, tetrahydrofuran and acetone were used for manufacturing equipment, spiked with drugs albuterol, loratadine and mometasone furoate and excipient lactose. Both rinse and swab samples were obtained. The quantitation of the pharmaceutical substances was demonstrated and the potential of using CAD for nonchromophore impurities, or contaminants was examined. Contaminants were obtained from manufacturing equipment. The analysis results were compared to those obtained by HPLC-UV.

An HPLC system (Waters Alliance 2695) was equipped with a PDA detector in line with a Corona CAD (ESA Inc.), a YMC Pack Pro C18 column, 50 mm × 4.6 mm, 3-µm particle size (YMC Co.) was used.[43] Moreover, an orbital shaker (New Brunswick Scientific) was used for the agitation of the swab samples. The mixture of 75:25 methanol:water was used as the mobile phase. Preparation of cotton balls for swab sampling was performed as follows: first sterile cotton balls were rinsing with methanol. Then a single cotton ball was placed into a 60-ml bottle and bottles were placed into a vacuum oven. A light nitrogen purge was added to remove the residual MeOH from the cotton balls. After this the bottles were removed from the oven, allowed to cool in a desiccator and then capped after cooling. Before swabbing 2 ml of acetone was added to the cotton ball.

In the research[43] the linearity of the APIs was examined and according the results the response could be treated as linear using both CAD and UV detector.[38] The response was linear over the usable concentration range from 10 ng to 400 ng on column. LOQs also were examined for each of the APIs and both detectors were able to achieve the criterion $S/N > 10$. For the all APIs limit of quantifications were discovered between 2 ng on column and 5 ng on column with CAD and between 3 ng on column and 6 ng on column using UV detection. The interference from wash solvents was less with CAD compared to UV detection at 205 nm. Both CAD and UV obtained acceptable recovery results but the CAD gave slightly better results. Recovery studies mimicked real surfaces found in commercial equipment with sampling techniques used in pharmaceutical cleaning assays (rinsing and swabbing). Lactose was an example for a poor or nonchromophore

compound and its analyse with CAD obviously demonstrated the better feasibility of the CAD compared to UV detection. The research shows that charged aerosol detection is a good alternative of detector for cleaning validation, specially when substances with low UV response is analysed.

6 Conclusions

Liquid chromatography is a popular technique for pharmaceutical analyses, like for example assay and impurities determination.[2,9] HPLC is actually primarily used in drug industry for evaluations of a large variety of samples and reversed-phase chromatography is the most common HPLC technique where the mobile phase is a polar and stationary phase is a nonpolar.

Methods are possible to speed up by using appropriated columns.[2,10,12] The conventional HPLC method can be shortened using short columns or column with small particle packing. The most effective way is to use the core-shell column. These columns do not generate high pressure and thus the HPLC instrument is suitable. By using recent small columns with core-shell packing faster separations can be provided compared to the columns of same size packing with fully porous particles. This is possible for both small and macromolecules. For example, the 5 cm long column with core-shell packing can achieve plate numbers N = 9 – 19,000 while the equally long and narrow bore column packed with fully porous particles can provide plate counts N = 7000 – 13,000 for small analytes. The most effective commercially available column is the Kinetex, which is packed with the core-shell type particles. Also auspicious packing solving is the 1.7 µm Eiroshell. Columns with sub-2 µm particles are general way of fast LC also, but these columns generate very high pressure.[10] Therefore, instrument must be UHPLC which stands high pressures, up to over 1000 bar. If the UHPLC technique system is available, most of the conventional LC methods can be transferred to extra fast separation LC methods without losing sensitivity or resolution in pharmaceutical analysis. When transferring method, comparable method parameters must be used to maintain equivalent separations.

Other options for faster liquid chromatography are monolithic columns and high temperature liquid chromatography, but they are quite rarely used.[16] Core-shell technology and UHPLC system have become more popular, due to for example higher performance compared monolithic columns. Also, many pharmaceutical compounds are sensitive to degradation in high temperatures so HTLC is not appropriate alternative in this case.

Several detectors are available that can be coupled with the liquid chromatography. The common detectors are UV/Vis detectors and PDA detectors. Also, the aerosol-based detectors are available like CAD, ELSD and CNLSD/NQAD. This thesis focused on the aerosol-based detectors and comparison of them because these detectors are universal; the response is for any analyte, not only for a certain class of compounds. Many studies have researched performance of various detectors. According the results of Vervoort *et al.*[30] CAD is more sensitive than ELSD but both were able to detect all compounds with sufficient accuracy and sensitivity. Both CAD and ELSD had non-linear response curves and they had to modify into linear one by a log-log transformation. Then linearity of CAD was better. Magnusson *et al.*[33] discussed several studies in their review article and they reached a conclusion that CNLSD/NQAD was much more sensitive than ELSD, but sensitivity between CNLSD/NQAD and CAD (or UV detection) varied. Olšovská *et al.*[35] developed the RP-UHPLC-NQAD method for determination of compounds with low absorption. Difference of response between detectors NQAD and UV was significant: NQAD was three times more sensitive than UV.

CAD is easy to use thus it is very applicable for both method development and routine analysis.[38] ELSD and CNLSD can be complex to operate and they are analyte dependent. CAD is generally more sensitive technique; it has higher detector response compared to ELSD. CAD was a little more sensitive than UV detector in the research of the cleaning validation by Snow and Forsatz.[43] Also, CAD obtained slightly better recovery results.

In conclusion, all aerosol-based detectors are practical solutions for detection of universal compounds and each one detected all pharmaceutical compounds with sufficient accuracy and sensitivity in the discussed researches. However, CAD and CNLSD/NQAD have better validation characteristics like accuracy and sensitivity than ELSD. But, CAD may be easier to apply than CNLSD/NQAD, which may be complex to operate and it may have tendency to be dependent on the analyte.

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