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QUANTITATIVE STRUCTURE RETENTION RELATIONSHIPS OF SELECTED ALPHA ADRENERGIC AND IMIDAZOLINE RECEPTORS LIGANDS IN THIN LAYER CHROMATOGRAPHY

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KVANTITATIVNI ODNOSI STRUKTURE I RETENCIONIH OSOBINA ODABRANIH LIGANADA ALFA ADRENERGIČKIH I IMIDAZOLINSKIH RECEPTORA U TANKOSLOJNOJ HROMATOGRAFIJI

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QUANTITATIVE STRUCTURE RETENTION RELATIONSHIPS OF SELECTED ALPHA ADRENERGIC AND IMIDAZOLINE RECEPTORS LIGANDS IN THIN LAYER CHROMATOGRAPHY

ABSTRACT

The concept of non-adrenergic imidazoline receptors (IRs) and their ligands was proposed based on a discovery that antihypertensive drug clonidine and its analogues exert their effect on the central nervous system by interaction with both, the α_2-adrenoreceptors (α_2-AR) and the imidazoline receptors. Further pharmacological studies allowed characterization of three subtypes of imidazoline receptors: I_1-, I_2- and I_3-IR. The second-generation agents, such as rilmenidine and moxonidine are more selective for I_1-imidazoline receptors and have produced fewer side effects than clonidine and other nonselective imidazoline receptors ligand.

The framework of this PhD dissertation was to determine retention parameters and create Quantitative Structure Retention Relationship (QSRR) models of selected alpha adrenergic and imidazoline receptors ligands as well as quantitative analysis of moxonidine and its impurities by Thin Layer Chromatography (TLC).

Retention behavior of 16 alpha adrenergic and imidazoline receptor ligands have been investigated by reversed-phase thin-layer chromatography (RP-TLC) on RP-18 and CN stationary phases using different mode of development (vertical and horizontal). Two different mobile phase systems consisting of methanol-water and tetrahydrofuran-ammonia-water were used in order to determine retention parameters (R_M^0, m, C_0) as a measure of the lipophilicity of the tested compounds. The experimentally determined retention constants were correlated with the log P values calculated with use of several methods. High correlations obtained between R_M^0 and logP values point out to the best chromatographic system for the estimation of lipohilicity of the tested compounds.

The QSRR modeling of 16 alpha adrenergic and imidazoline receptor ligands was performed with use of the PLS regression in order to reveal the most influential factors governing the retention. In the QSRR study, experimentally obtained retention parameters (R_M^0) in the three different chromatographic systems were used as dependent variables, while the computed molecular parameters of the examined compounds were used as

independent variables. The developed QSRR models were tested by use of the cross-validation and external test set prediction. The created models indicated that apart from lipophilicity, constitutional descriptors, P_VSA-like descriptors and hydrogen bonding properties of the tested compounds were important for the retention behavior in the RP-TLC systems. On the basis of obtained results, the developed QSRR models can be successfully used as predictive tools for evaluation of the R_M^0 values of the related guanidine and imidazoline derivatives.

Development and validation of thin-layer chromatographic method for simultaneous determination of moxonidine and its four impurities have been performed using chromatographic plates precoated with silica gel 60 F_{254} and methanol-toluene-dichloroethane-ammonia 2:3:3:0.1 (v/v/v/v) as mobile phase. The developed chromatographic plates were scanned at the wavelengths 260 and 280 nm. The proposed TLC method fulfilled all validation requirments (linearity ($r \geq 0.998$), accuracy (recovery: 90.10 % - 107.63 %), precision, sensitivity (LOQ of impurities 20 ng $band^{-1}$ equivalent to the 0.12 % impurity level) and robustness) which affirm the suitability of the method for quantitative analysis of moxonidine and its impurities in available commercial pharmaceutical dosage forms.

Keywords: imidazoline receptors, alpha adrenergic receptors, lipophilicity, PLS, QSRR, TLC, impurities, moxonidine, method validation.

Scientific field: Pharmacy

Narrow scientific field: Pharmaceutical-medicinal chemistry and structural analysis

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KVANTITATIVNI ODNOSI STRUKTURE I RETENCIONIH OSOBINA ODABRANIH LIGANADA ALFA ADRENERGIČKIH I IMIDAZOLINSKIH RECEPTORA U TANKOSLOJNOJ HROMATOGRAFIJI

REZIME

Koncept neadrenergičkih imidazolinskih receptora (IRs) i njihovih liganada je predložen na osnovu saznanja da antihipertenzivni lek klonidin i njegovi analozi ostvaruju svoj efekat na centralni nervni sistem interakcijom sa dva tipa receptora, α_2-adrenergičkim (α_2-AR) i imidazolinskim receptorima. Dalje farmakološke studije omogućile su klasifikaciju tri podtipa imidazolinskih receptora: I_1-, I_2- i I_3-IR, a noviji lekovi koji pripadaju drugoj generaciji agenasa, kao što su rilmenidin i moksonidin, su selektivniji za I_1-imidazolinske receptore i imaju manje neželjenih efekata u poredjenju sa klonidinom i drugim neselektivnim ligandima.

U ovoj doktorskoj disertaciji primenom metode tankoslojne hromatografije (TLC) određeni su retencioni parametric, a zatim formirani QSRR (*Quantitative Structure Retention Relationship*) modeli za 16 odabranih liganada alfa adrenergičkih i imidazolinskih receptora; i izvršena je kvantitativna analiza moksonidina i njegovih nečistoća.

Retenciono ponašanje 16 liganada alfa adrenergičkih i imidazolinskih receptora ispitano je metodom reverzno-fazne tankoslojne hromatografije (RP-TLC) na RP-18 i CN-stacionarnim fazama primenom vertikalnog i horizontalnog načina razvijanja hromatograma. Dva različita sistema mobilnih faza koje čine metanol-voda i tetrahidrofuran-amonijak-voda su primenjena sa ciljem da se odrede retencioni parametri (R_M^0, m, C_0) koji se mogu koristiti kao mera lipofilnosti testiranih jedinjenja. Eksperimentalno određene retencione konstante su korelisane sa log P vrednostima izračunatim primenom nekoliko metoda. Na osnovu visokih korelacija dobijenih između R_M^0 i log P vrednosti izdvojeni su najbolji hromatografski sistemi za procenu lipofilnosti ispitivanih jedinjenja.

Primenom parcijalne regresije najmanjih kvadrata (PLS) formirani su QSRR modeli u kojima su odabrani molekulski parametri sa najvećim uticajem na retenciono ponašanje ispitivaih jedinjenja. U QSRR studiji, eksperimentalno dobijeni retencioni parametri

(R_M^0) određeni primenom tri različita hromatografska sistema su korišćeni kao zavisno promenljive dok su izračunati molekulski deskriptori korišćeni kao nezavisno promenljive. Formirani QSRR modeli su validirani primenom ukrštene validacije i eksterne validacije i ukazali su da pored lipofilnosti, konstitucioni deskriptori, *P_VSA-like* deskriptori i sposobnost testiranih jedinjenja da učestvuju u građenju vodoničnih veza predstavljaju parametre važne za retenciono ponašanje u RP-TLC sistemima. Dobijeni statistički podaci potvrdili su da se formirani QSRR modeli mogu uspešno primeniti za pouzdano predviđanje R_M^0 vrednosti srodnih derivate gvanidina i imidazolina.

Metoda tankoslojne hromatografije, u kojoj je silika gel 60 F_{254} korišćen kao stacionarna faza, a smeša metanol-toluen-dihloretan-amonijak 2:3:3:0.1 (v/v/v/v) kao mobilna faza, je optimizovana i validirana za istovremeno određivanje moksonidina i njegove četiri nečistoće. Razvijene hromatografske ploče su skenirane na talasnim dužinama od 260 i 280 nm. Predložena TLC metoda je zadovoljila sve validacione zahteve (linearnost, $r \geq 0{,}998$; tačnost (*recovery* 90,10 % - 107,63 %), preciznost, osetljivost (LOQ 20 ng po traci što odgovara sadržaju nečistoća od 0,12 %) i robusnost), čime je potvrđena njena pogodnost u kvantitativnoj analizi moksonidina i njegovih nečistoća u komercijalno dostupnim farmaceutskim oblicima.

Ključne reči: imidazolinski receptori, alfa adrenergički receptori, lipofilnost, PLS, QSRR, TLC, nečistoće, moksonidin, validacija.

Naučna oblast: Farmacija

Uža naučna oblast: Farmaceutska-medicinska hemija i strukturna analiza

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CONTENT

LIST OF ABBREVIATION

AR - Adrenergic Receptors

ANN - Artificial Neural Network

B3LYP – Beck 3-parameter, Lee, Yang and Parr

BP - British Pharmacopoeia

CNS - Central Nervous System

CDS - Clonidine Displacing Substance

DFT - Density Functional Theory

EP - European Pharmacopoeia

GC - Gas Chromatography

HILIC - Hydrophilic Interaction Liquid Chromatography

ICH - International Conference on Harmonisation

IR - Imidazoline Receptor

I_1-IR/α_2-AR – Imidazoline and α_2-adrenergic receptor ligands

LSER - Linear Solvation Energy Relationship

LOO-CV- Leave-One-Out Cross-Validation

LOD - Limit of Detection

LOQ - Limit of Quantification

MLR - Multiple Linear Regression

MD - Migration Distances

PLS - Partial Least Squares

PCA - Principle Component Analysis

QSRR - Quantitative Structure-Retention Relationships

QSPR - Quantitative Structure-Property Relationships

QSAR - Quantitative Structure-Activity Relationships

RP-TLC – Reversed-Phase Thin Layer Chromatography

RP – HPLC - Reversed Phase-High Performance Liquid Chromatography

RMSEE - Root Mean Square Error of Estimation

RMSEP - Root Mean Square Error of Prediction

RSD - Relative Standard Deviation

SIMCA - Soft Independent Modeling of Class Analogy

TLC - Thin Layer Chromatography

UPLC - Ultra Performance Liquid Chromatography

VIP - Variable Importance in the Projection

1. Introduction

1.1. Planar chromatography

The first scientists discovered and used thin-layer chromatography (TLC) are Schraiber and Izmailov in 1939. [1] Schraiber separated medicinal component on unbounded alumina or other adsorbents spread on glass plates. Because they applied drops of solvent to the plate containing sample and sorbent layer, the procedure was called drop chromatography. Meinhard and Hall in 1949 used binder to adhere alumina to microscope slides, and these layers were used in the separation of inorganic ions with the use of drop chromatography. This method was called surface chromatography. In the 1950, Kirchner and colleagues at the U.S department of agriculture performed TLC as we know it today. They used silica gel held on glass plates with the aid of a binder. Plates were developed with conventional ascending procedures used in paper chromatography. Kirchner also coined the term "chromatostrip" which for the first time contained fluorescence indicator. Stahl introduced the term "thin-layer chromatography" in the late of 1950s. Quantitative thin layer chromatography was introduced by Kirchner in 1954 when they described elution method of determination of biphenyl in citrus fruits. Densitometry in thin layer chromatography was reported in 1960 using commercial densitometers such as the Photovolt and Joyce Loebl Chromascan. High Performance Thin Layer Chromatography (HPTLC), instrumental HPTLC, and over pressured layer chromatography (OPLC), were introduced in the late 1970s. [2]

1.1.1. Theory of planar chromatography

In planar chromatography, adsorbent is coated onto a solid support as a thin layer (about 0.25 mm thick). In many cases, a small amount of a binder such as plaster of paris is mixed with the adsorbent to facilitate the coating. Many different solid supports are used, including thin sheets of glass, plastic, and aluminium. The mixture (A and B) to be separated is dissolved in a solvent and the resulting solution is spotted onto the thin layer plate near the bottom. A solvent, or mixture of solvents, called eluent, is allowed to flow up the plate by capillary action. At all times, the solid will adsorb a certain fraction of each component of the mixture and the remainder will be in solution. A substance that is strongly adsorbed (A) will have a greater fraction of its molecules adsorbed at any time, and thus any one molecule of (A) will spend more time sitting still and less time moving. In contrast, a weak adsorbed substance (B) will have a smaller fraction of its molecules

adsorbed at any one time, and hence any one molecule of (B) will spend less time sitting and more time moving. Several factors determine the efficiency of chromatographic separation. The adsorbent should show a maximum of selectivity toward the separated substances so that the difference in rate of elution will be large. For the separation of any mixture, some adsorbents may be too strongly adsorbing or too weakly adsorbing. The eluting solvent should also show a maximum of selectivity in its ability to dissolve or adsorb the separated substances. The fact that one substance is relatively soluble in a solvent can result in its being eluted faster than another substance. However, a more important property of the solvent is its ability to be itself adsorbed on the adsorbent. If the solvent is more strongly adsorbed than the separated substance, it can take their place on the adsorbent and all the substances will flow together. If the solvent is less strongly adsorbed than any of the components of the mixture, its contribution to different rates of elution will be only through its difference in solvent power toward them. If, however, it is more, strongly adsorbed than some components of the mixture and less strongly than others, it will greatly speed the elution of those substances that it can replace on the adsorbent, without speeding the elution of the others. [3]

1.1.2. Types of planar chromatography

There are two main branches of planar chromatography: paper chromatography and thin-layer chromatography.

Paper chromatography

Paper chromatography is a technique that involves placing a small dot or line of sample solutions onto a strip of chromatography paper. The paper is placed in a container with a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture, which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel further if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

Thin-layer chromatography

Thin layer chromatography is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a

flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. TLC can be used for quantitative analysis. TLC and HPTLC are very simple and economical methods of analysis. [4] Their field of application covers all classes of substances with the exception of volatile and gaseous substances and can be extended easily to the preparative scale by using thicker layers (preparative layer chromatography, PLC). The separated substances depending on their optical properties, can be detected, identified and quantified in visible, infra-red or UV light and in certain cases only after derivatization with a suitable reagent.

On the other hand thin-layer chromatography can be coupled with other spectroscopic methods and also with other analytical and preparative separation methods. Established coupling of separation methods include: LC-TLC (Liquid Chromatography-Thin Layer Chromatography), HPLC-HPTLC (High Performance Liquid Chromatography- High Performance Thin Layer Chromatography), HPLC-AMD (High Performance Liquid Chromatography-Automated Multiple Development), TLC-Electrophoresis. Example of the successful coupling of planar chromatography with spectroscopic methods include: TLC/HPTLC-UV/VIS, TLC/PTLC-Fluorescence (Thin Layer Chromatography/Preparative Thin Layer Chromatography- Fluorescence), TLC/HPTLC-FTIR (Thin Layer Chromatography/High Performance Thin Layer Chromatography-Fourier-Transform Infra – Red), TLC/HPTLC-RAMAN (Thin Layer Chromatography/ High Performance Thin Layer Chromatography-Raman spectroscopy),

TLC/HPTLC-SERRS (Thin Layer Chromatography/ High Performance Thin Layer Chromatography - Surface - Enhanced Resonance Raman Scattering)

TLC/HPTLC-MS (Thin Layer Chromatography/ High Performance Thin Layer Chromatography-Mass Spectrometry). [4]

The main advantages of TLC methods that other techniques never achieve are: [5]

1. Simple and easy to use, and need no professional knowledge for throughout, and rapid, low cost analysis.

2. Can analyze complex or dirty samples without pre-cleaning due to the use of disposable adsorbent layer.

3. Both, highly polar and non–polar impurities can be detected on the adsorbent layer; even if sitting on the origin or traveling in the eluent front.

4. Can combine and consecutively use different modes of evaluation, allowing identification of impurities having different light absorption characteristics or different colors after spraying.

5. Are well suited for comparison of impurity - profiles of samples of different origin because of the possibility of simultaneous analyzing different samples

1.1.3. Sample application in planar chromatography

Sample application is the first step in the workflow of planar chromatography and it affects significantly the quality of the result at the end of the process. The choice of the application technique and the device depend on the requirements of precision, sample volumes, number of analyses and the desired grade of automation. Spot wise sample application using a fixed volume capillary is the simplest way. Sample volumes of 0.5 to 5 μL can be applied as spots onto conventional layers without intermediate drying, on HPTLC layers it is up to 1 μL per spot.

Linomate V (Camage, Muttenz, Switzerland) is one of the most widely used automatic TLC sampler, in which samples are applied onto TLC plates in the form of bands or horizontal lines with nitrogen or compressed air applicator for precise qualitative and quantitative analysis. The application of samples in the form of narrow bands or horizontal lines, provides significantly larger volumes which result in the best resolution of sample in a given chromatographic system. [2, 5]

1.1.4. Method development in planar chromatography

The most common method of developing a chromatogram is to put the plate in a chamber, which contains enough quantity of developing solvent. The lower end of the plate should be immersed several millimeters. Driven by capillary action the developing solvent moves up the layer until the desired running distance is reached and chromatography is stopped. [2, 3, 6, 7, 11] There are several different types of developing chamber.

Classical developing chamber

Selection of the suitable chamber is done during method development and generally follows practical considerations such as which chamber is available, or which one has been used in the past if a results comparison is to be made. However, a focus should also be on economic aspects such as time requirement and solvent consumption.

Horizontal developing chambers

These chambers are characterized with economic aspects, flexible and reproducible in operation. Although designed for applications where the plate is developed from two sides, they are also suitable for single-sided developments in unsaturated, saturated and sandwich configuration as well as for pre-conditionin of HPTLC plates.

Automatic developing chamber

This instrument does not only eliminate any effects of the operator when introducing the plate into a saturated chamber but also the activity of the layer prior to start of chromatography can be set. In addition, drying of the chromatographed plate is rapid and complete. For development a conventional 20 x 10 cm Twin Trough Chamber is used. This way chamber geometry and chromatographic conditions of already existing analytical procedures can be retained, but environmental and operational effects are standardized. In case when the sample contains polar and non-polar components which must be separated in the same analysis, the principle of Automated Multiple Development (AMD) can be employed. Development is performed on the basis of a solvent gradient from polar to non-polar over several steps with intermediate drying. [7, 8]

1.1.5. Detection in planar chromatography

The chromatogram is evaluated under white or ultraviolet fluorescent light in visible regions or by densitometer scanner for quantitative determinations. A common practice in TLC analysis include the use of plates containing fluorescence indicator, excited by UV light, usually at 254 or 366 nm, resulting in green or blue background. Compounds absorbing at 254 or 366 nm cause fluorescence quenching, appearing as dark spots on the white or colored background.

Detection in scanning densitometry

Classical densitometry uses monochromatic light and a slit of selectable length and width to scan the tracks of a chromatogram, measuring the diffusely reflected light or fluorescence. The CAMAG TLC Scanner uses the entire spectral range from 190 to 900 nm with high spectral selectivity for data acquisition. Absorption spectra for substance identification and for selection of the most suitable measurement wavelength can be

recorded within this range. The strengths of classical densitometry are the spectral resolution of the light source and the higher reproducibility of quantitative determinations. Fluorescence measurement has several advantages such as, greater selectivity, higher sensitivity, linear relationship between fluorescent intensity and compound concentration, and band shape not influencing the signal. To enhance fluorescence, the plate may be treated with some reagent e.g exposure to ammonia vapors. [3, 9]

1.1.6. Stationary phases in planar chromatography

Stationary phases used in modern TLC (adsorption chromatography) are mostly solids and their impact on retention (known as retardation, or slowing of solute migration) is by mechanism of adsorption. Thus TLC stationary phases can correctly be regarded as adsorbents. In chromatography however one prefers to employ a somewhat broader notion of 'adsorption', as better reflecting the fact that the process of adsorption-although obviously occurring on the adsorbent surface-often occurs not only on the outer solid-liquid interface of the solid bed particles but also on the inner surfaces of micropores situated within these particles. Adsorbents performance, their capacity to interact effectively with analyte is commonly known as adsorbent activity. The more active adsorbent is, the greater is interaction with the analyte molecule and hence the more pronounced is retardation of their migration through the solid bed. It is experimentally well established that surface area of adsorbents are not equipotential but consists of sub-areas (usually single atoms or groups of atoms) largely differentiated in particular respect. These sub-areas on the adsorbents surface characterize nature of adsorption. The activity of an adsorbent (adsorbent with uniform chemical characteristics e.g silica or alumina) depends on three properties:

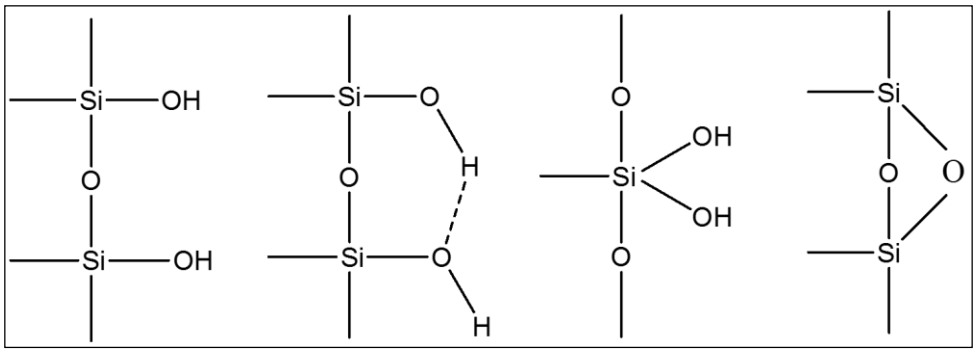
1. Specific surface area-which depends on the chemical structure of the adsorbent and on technology used in its manufacture.
2. The density of free (i.e. unoccupied by water molecules) active centers per unit adsorbent surface area. It is depends on chemical structure of the adsorbent and on the number of water molecules occupying its active centers. This property can also be measured and expressed numerically.

3. The energy of intermolecular interactions between a solute molecule and a given type of adsorbent active center - which depends as much on the chemical nature of the adsorbent as on the solute itself. Adsorbents used in TLC are classified in several different ways, depending on the classification criterion used. [9] The most practical classification of adsorbents depend on their chemical structure.

Adsorbents with uniformchemical characteristics

These are usually inorganic compounds (e.g. silica, alumina, florisil) and some organic materials (e.g. cellulose powder, chitin, polyamides). Since the beginning of thin-layer chromatography adsorbents with uniform (non-combined) chemical characteristics have indisputably predominated in terms of practical applications: it is therefore hardly to overestimate their relative importance. Nowadays they have been used as industrially pre-coated layers. In addition to glass plates, plastic and alumina sheets are also used as supports for pre-coated layers.

Silica gel is probably the most important single substance involved in chromatography today. It is not only used as a polar stationary phase per se, but it also forms the basic skeleton upon which the so called "bonded phases' are manufactured. Chemically all silica gels are silicon dioxides. Each silicon atom is surrounded by four oxygen atom to form a tetrahedron. At the surface of the silica gel the free oxygen valences are connected either to hydrogen (Si-OH, silanol groups) or to another silicon atom (Si - O- Si, siloxane groups). Silica gels used in thin-layer chromatography are porous synthesized materials. Irrespective of the details of the technology used in their manufacture the density of silanol groups is uniform for all types of silica gel, approximately 8 μmol m^{-2}. To a convenient first approximation the active centers of silica gel can be regarded as identical, and thus the surface structure can be thought of as very simple. In reality, the silanol active centers can differ slightly, depending on whether they occur as isolated silanols, or as neighboring (i.e. vicinal) or geminal silanols as illustrated schematically in Figure 1.



a) b) c) d)

Figure 1. Functional groups on the surface of silica: a) free (isolated); b) vicinal (neighboring); c) geminal silanols groups and d) siloxane groups.

Silica is a polar sorbent with hydrophilic properties, hence water molecule from the air are easily absorbed on its surface. Water overlay ratio significally influence on silica gel's activity. Intermolecular interactions between surface active centers of silica gel and analyte molecules basically result from the formation of hydrogen bonds between the sylanol hydrogen atom and basic sites on the analytes (e.g. N, O and S heteroatom and p-electrons) as shown as: ≡Si-OH·····Y-R, where ≡Si-OH is the silanol active center, and R-Y is the molecule of analyte (with Y denoting the basic center of the analyte). The main field of application of silica gels as stationary phase is in adsorption chromatography. Because silica gel usually performs as very active adsorbents, stringent requirements are placed on the mixed mobile phase used with this material. Most fields of application of this stationary phase are for determination of alfatoxins, alkaloids, lipids, pesticides. [10] Other inorganic and organic sorbents such as alumina, magnesium silicate, diatomaceous earth (kieselguhr), cellulose and cellulose derivatives, and polyamides have found only limited application in TLC, although their separation capacities differ from those of adsorption and reversed- phase layers based on silica.

Chemically modified adsorbents

Adsorbents with combined chemical characteristics are composed of a matrix with the ligands chemically bonded to its surface. The most important adsorbents with combined chemical characteristics are the so-called aliphatic chemically bonded phases. Their importance results from broad scope of practical separations possible by use of these materials. Porous silica gel is used as matrix and organic functional group (e.g. methyl

(RP-2), octyl (RP-8), octadecyl (RP-18)) as ligands. These phases can be divided in two classes: hydrophobic, i.e. non-polar and hydrophilic, i.e. polar (Figure 2). The most important commercially available hydrophobic ligands are methyl (RP-2), octyl (RP-8), octadecyl (RP-18). The hydrophobicity of the respective adsorbents depends not only on the type of ligand, but also on density of coverage of the silica matrix. The use of these adsorbents almost automatically forces use of aqueous mobile phase resulting in reversed-phase chromatographic system. The mechanism of solute retention on these adsorbents is usually more complex than for adsorbents with uniform characteristics, because of highly pronounced heterogeneity of stationary phase surface of organic chemically bonded adsorbents. Free (non-bonded) silanol groups originated from silica gel matrix and the organic chemically bonded hydrophobic or hydrophilic ligands can co-exist simultaneously on such surface. Thus the molecule of analyte can interact by hydrogen-bonding with silanol group or polar group and can interact through dispersive force with hydrophobic ligands and with the alkyl moieties of hydrophilic ligands. Because of highly pronounced heterogeneity of stationary phase surface of chemically bonded adsorbents there is no uniform mechanism of solute retention. The mixed mechanisms of analyte generated on this way have specific impact on the quality of chromatographic separations. The hydrophobic combined adsorbents are applicable in TLC of alkaloids, peptides antibiotics, carboxylic and sulfonic acids and in ion-pair chromatography, on the other hand hydrophilic are used for phenols, steroids. [6, 9]

The derivatization of silica gel with cyano propyl group produce a hydrophilic stationary phase (cyano modified silica), that have been used for the separation and extraction of very polar compounds. [2, 6, 11] The main advantage of cyano phase is it''s ability to provide chromatographic retention both in normal such as amine compouds and reversed – phase separation such as flavonoids, and steroids due to their moderate polarity. [12, 13]

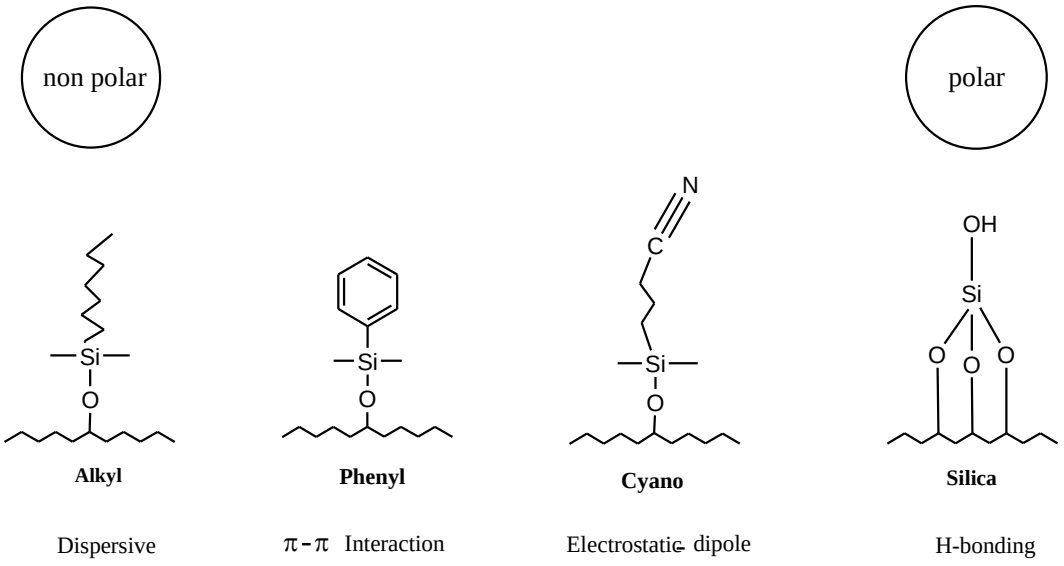


Figure 2. Different types of stationary phases

1.1.7. Mobile phases in planar chromatography

Solvent system is a liquid mobile phase composed of one or more miscible solvents. The solvent system competes with the dissolved analyte for the active sites on the sorbent and must be carefully selected to achieve a good separation of individual components. The choice of the optimum mobile phase composition is often the most difficult and most time consuming procedure involved in the development of a TLC analytical method. Even though the mobile phase may be selected on a rational basis, in the end, the composition of the optimum solvent mixture will always need to be confirmed by careful experiment. Solvent systems are selected by considering the equilibrium between the solvent, and solutes, and the sorbent layer. The selection of a solvent system must take into consideration several factors, the most important being a good separation of the component in the mixture. The choice of the mobile phase depends on the nature of the compounds to be separated. [14] The interactions between the analyte-mobile phase or the analyte-sorbent may be determined by the number and nature of the functional groups in the analyte. To obtain the greatest selectivity, the properties of the mobile and stationary phases should be as different as possible. If stationary phase is polar (bare silica) the mobile phase should be non-polar or slightly polar and vice versa. Separations will become less time consuming with increased practical experience and as chromatography skills are developed and improve. Other factors considered in the selection of a solvent

system may include the cost, availability, quality, toxicity, volatility, and miscibility of the solvent or solvents chosen. Simple systems of one or two solvents are preferred over complex mixtures of several solvents. The purest grade solvents should be used since any impurities can greatly affect the selectivity and reproducibility of the separation. [9, 11]

Solvent classification

Synder classified common solvents according to their polarity (P') and their selectivity or relative ability to engage in hydrogen bonding or dipole interaction.

Polarity is widely used in chromatography as measure of capability of compounds to interact between each other. Relative polarity of solvents or solvent mixtures can be quantified by solubility parameter (δ), or by index of polarity (P') but all these quantification of polarity aren't quite adequate.

Solubility parameter (δ) could be defined by following equation**:**

$$\delta = \left(\frac{\Delta E}{V} \right)^{1/2} \qquad (1)$$

Where ΔE represents molar energy of evaporization, and V molar volume. For the mixture of solvents solubility parameter could be calculated by equation:

$$\delta_m = \sum_i \delta_i \times V_i \qquad (2)$$

Where δm is solubility of a mixture of solvent, Vi is volume fraction, and δi is solubility parameter of component i of a mixture.

Index of polarity (P') is experimental determined by gas chromatography and represents coefficient of distribution three test solution on large number of stationary phases. [15]

Eluotropic series. The term eluotropic series was coined by Trappe. [16] In eluotropic series the solvents are ordered depending on their power to carry a given compound through stationary phase (e.g silica gel). The order of solvents runs in the order of their polarity. The series is useful when selecting a solvent for a particular separation where more polar compounds will normally require a more polar solvent. The interaction

between a particular solvent and a sorbent is based on the eluting power of the solvent, which is defined by the solvent strength parameter, ε^0.

From Snyder textbook a more precise definition was accepted: the eluent strength of a solvent is the standard free energy of adsorbed solvent molecules in standard state. [17] A larger ε_0 indicate a greater interaction between the solvent and the sorbent. In most cases, the strength of a solvent mixture will be intermediate between the strength of the two or more components of the mixture.

Solvent selectivity triangle

The most enduring approach to solvent classification used by LC chromatographers, especially doing small molecule in normal and reverse phase separation, is L.R. Snyder's solvent strength and solvent triangle classification method. In published papers from 1974-78, Snyder reported classification about 75 solvents bases on their interactions with three different solutes:

1. Ethanol (e)-assumed to have proton donating and hydrogen-bonding character
2. 1, 4-dioxane (d)- assumed to have proton accepting character
3. Nitro methane (n)-having permanent dipole interactions. [18]

Each of their individual properties are weighted and summed according to:

X total = Xe + Xd + Xn = 1.00 (3)

Where Xe = log(K'g)e / P' (the solvents relative strength to interactive with ethanol and receive a proton, so a proton acceptor).

Xd = log(K'g)d / P' (the solvents relative strength as a proton donor).

Xn = log(K'g)n / P' (the solvents relative strength for permanent dipole interactions).

Snyder pointed out that 'polarity normalization' results in a distinction between solvent strength and solvent selectivity. The plot using triangular coordinates is displayed in following Figure 3.

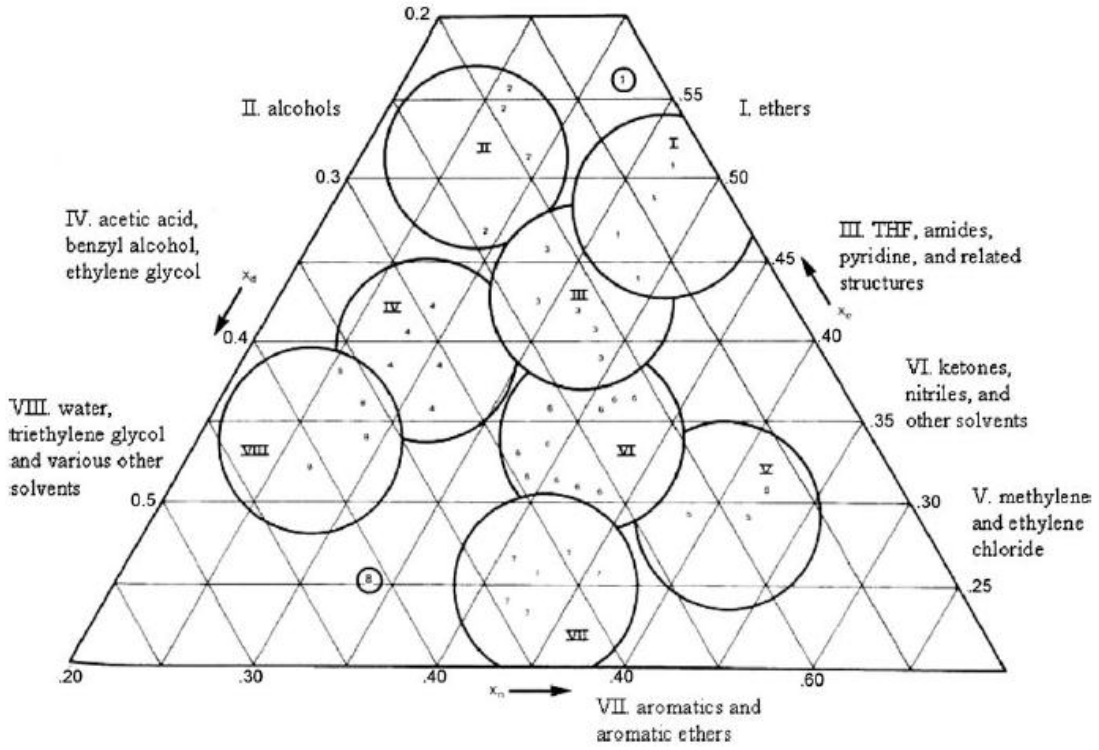


Figure 3. Snyder's selectivity triangle**.** [19]

Solvents grouped in same region of triangle will have similar selectivity whereas solvents from other groups will have different selectivity even if their solvent strength is similar. Solvents are classified into eight groups (Table 1.).

Table 1. Solvent classification according to Snyder

Group	**solvents**
I	Aliphatic ethers
II	Aliphatic alcohols
III	Pyridine derivative, THF, sulfoxides
IV	Glycols, acetic acids
V	Dichloromethane, 1,2-dichloroethane
VI	a-Aliphatic ketones and esters, dioxane b-Sulfones, nitriles
VII	Aromatic hydrocarbons, halosubstituted aromatic hydrocarbons, nitro compound, aromatic ether
VIII	Fluoroalkanols, water

Chromatographic system can be classified as normal or reversed phase ones based on polarity of stationary and mobile phase.

Normal-phase chromatographic system

It is consists of relatively polar material with a high specific surface area as the stationary phase silica being the most popular, but alumina and magnesium oxide are also often used. The mobile phase is relatively non-polar (heptane to tetrahydrofuran). The different extents to which the various types of molecules in the mixture are adsorbed on the stationary phase provide the separation effect. A non-polar solvent such as hexane elutes more slowly than a medium-polar solvent such as ether. Polar compounds show a greater retention because the specific interactions between sorbent and analyte are more pronounced.

Reversed-phase chromatographic system

It is consists of very non-polar stationary phase and relatively polar (water to THF) mobile phase. A polar solvent such as water elutes more slowly than a less polar solvent such as acetonitrile. The most commonly used stationary phases in reversed-phase conditions are alkyl-modified silica gels (e.g., dimethyl, octyl-, octadecyl-modified silica gel) but other sorbents, such as cyanopropyl, or aminopropyl modified silica gels are also applied. Highly polar compounds are predominantly separated by reversed-phase chromatography.

1.2. Retention parameters in planar chromatography for estimation of compounds lipophilicity

Chromatographic retention parameters (R_M^0, R_f, C_0) obtained by RP-TLC are widely used in medicinal chemistry and molecular pharmacology as a measure of lipophilicity, instead of reference lipophilicity parameter, log *P*. Lipophilicity of a compound is one of the most important physicochemical properties which influence drug absorption, distribution, metabolism and elimination. Our previously performed QSAR (Quantitative Structure-Activity Relationship) studies for the set of imidazoline receptor ligands revealed that lipophilicity of the drugs plays an important role, enhancing the I_1-IR binding affinity and I_1-IR/α_2-AR selectivity.[20, 21] Therefore, lipophilicity of a drug or a drug candidate is a very important physicochemical property, examined in the initial phase of drug discovery.

Lipophilicity is usually characterized through the logarithm of the octanol-water partition coefficient, log *P*, introduced into medicinal chemistry by Hansch and Fujita. A traditional approach for the determination of lipophilicity of molecule is the so called shake-flask method. [22, 23] Tediousness of determinations and limited inter-laboratory reproducibility of log *P*, on one hand, and the observation of linear relationship between log *P* and chromatographic retention parameters (R_M), on the other hand, gave rise to the substitution of the former by the readily available chromatographic data. [24] Nowadays, chromatographic methods such as RP-HPLC, and TLC are known as a unique methods [24, 25] which can yield a great amount of quantitatively comparable, precise and reproducible retention data for large sets of structurally different compounds which can be correlated with their physicochemical and biological properties giving appropriate QSPR (Quantitative Structure-Property Relationship) and QSAR models. [25-28] Lipophilicity and acidity of moxonidine and those of structurally similar imidazolines and oxazolines have been evaluated with aid of different separation techniques, HPLC [29] and TLC. [26] This evaluation was based on measuring of retention behavior of the compound of interest in the employed separation systems.

The most common retention parameters in TLC that were used for estimation of compounds lipophilicity are R_M^0 and C_0 which can be derived from the retention behaviour of compound.

Retention parameter (R_M) is derived by the retardation factor (R_f) according to the Bate-Smith and Westall equation: [30]

$$R_M = \log (1/ R_f - 1) \qquad (4)$$

Where R_f is calculated on the basis of migration distance of compound and the solvent front. As the retention behavior of investigated substances (R_M value) depends linearly on the concentration of the organic modifier in the mobile phase, the value is extrapolated to pure water as mobile phase according to equation:

$$R_M = R_M^0 + m\varphi \qquad (5)$$

Where φ represents the concentration of the organic component in the mobile phase and m is the slope, which indicate the rate at which the solubility of the solute in the mobile phase increases with changes in its composition. Lipophilicity measured as R_M^0,

represents the relative affinity of different compounds for the non-aqueous environment in the biological system. R_M^0 is the value of R_M in pure water, and therefore it could reflect the dependence of the hydrophobic properties of investigated compounds on its structure. [26, 31]The effect of solvent pH on R_M value was described by Mannhold and co-workers, and found that in case of low organic modifier content in the mobile phase, the effect of solvent pH on R_M is negligible. But in case of higher organic modifier concentration, the polar adsorption become more pronounced and effect of pH on R_M become strong in the case of basic solute. The most commonly used organic modifiers are methanol, acetone, and acetonitrile. Less frequently dioxane or tetrahydrofurane are used. Silica gel layers, impregnated with either a non-polar or an alkyl- modified (octadecyl) are usually used as stationary phase. [24]

Another chromatographic parameters that are used as hydrophobicity parameter is C_0, which have been introduced in HPLC [32, 33] and TLC. [34] It is calculated as the ratio of the intercept and slope:

$$C_0 = - R_M^0/m \qquad (6)$$

Where C_0 represent the volume fraction of the organic modifier in mobile phase for which the distribution of the solute between the two phases is equal, i.e $R_M = 0$ $R_f = 0.5$. Calculated lipophilic parameter C_0 can be used in the same manner as R_M^0 values.

Literature searching revealed that the satisfactory correlation between hydrophobicity parameters (R_M^0 and C_0) determined by thin layer chromatography and calculated log *P* as a standard measure for lipophilicity, indicated the suitability of this parameter as a measure of lipophilicity of the newly synthesized compound. [26, 34, 35]

1.3. Quantitative analysis in planar chromatography

In-situ evaluation in planar chromatography started in 1962 especially in the field of phytochemistry. In-situ scanning densitometry is the most commonly used method of quantification and involves plotting the absorbance or fluorescence of light from a scanned lane of the TLC plate. The plate is scanned with a beam of light and a detector. The intensity of the light reaching the detector changes in the presence of a separation zone. This change is a function of the amount of analyte present in the separation zone. The amount of any given component in an unknown sample is determined by comparing a plot of its separation zone to a standard calibration curve made from plots of reference

materials chromatographed on the same plate. This method requires appropriate equipment. [2, 3, 7, 14, 36] The satisfactory use of quantitative analytical method require other data evaluation procedures i.e application of validation procedure according to International Conference on Harmonisation (ICH) Guideline in the term of specificity, detection and quantification limit (LOD and LOQ), accuracy, precision, linearity, and robustness. [37, 38]

Thin layer chromatography is an important alternative to liquid chromatography in the quantitative analysis of pharmaceutically active substances and their degradation products. Thin layer chromatography have been successfully applied for determination of tetryzoline, [39] tizanidine, [40] harmine, and harmaline.[41] By applying chemometric approach in TLC analysis is possible that during the method optimization, using suitable experimental design methodology based on a small number of well-planned experiments, the most significant chromatographic parameters as well as their interactions influencing retention behavior of the active substance and its impurities can be identified and quantified thus giving robust and reliable methods that can be applied in quantitative analysis. Chemometric approach for the method optimization includes few important steps such as: definition of the objective of optimization, selection of important chromatographic factors that will be optimized and theirs levels, selection of experimental design and chromatographic response, and finally creation of mathematical model and identification of the optimal chromatoghraphic conditions. Types of the experimental design used in the process of optimization include the full factorial design, central composite design, Box-Behenken design, D-optimal design, etc. Which one will be selected depends on type of tested factors and their interval of variation, as well as of an acceptable number of experiments and analysts' expectations. [42, 43]

1.4. Alpha adrenergic and imidazoline receptors ligands

Adrenergic receptors

The adrenergic receptors or adrenoceptors (ARs) are a class of G protein-coupled receptors that are targets of the catecholamines, especially norepinephrine (noradrenaline) and epinephrine (adrenaline). There are two types of adrenergic receptors, α and β, with several subtypes. α- receptors have the subtypes α_1 (a G_q coupled receptor) and α_2 (a G_i coupled receptor).

Alpha adrenergic agonists

An alpha adrenergic agonist is a drug that selectively stimulates α-ARs (α_1 or α_2).

Alpha$_1$- agonists are chemically derivatives of 2-arylakyl imidazoline, that characterized by presence of a one carbon bridge between C-2 of the imidazoline ring and a phenyl ring. The presence of *ortho*-lipophilic groups on the phenyl ring are important for alpha-activity, while a *meta* or *para*-lipophilic substituents on the phenyl ring may be important for the alpha1 selectivity. Due to the basic nature of imidazoline ring (pK_a=10-11), these drugs at physiological pH exist in an ionized form**.** Large doses of these drugs (oxymetazoline) may cause hypotension because of a central clonidine-like effect. [44, 45] Selected examples: xylometazoline, oxymetazoline, naphazoline, tetrahydrozoline and tramazoline are presented on the Figure 4.

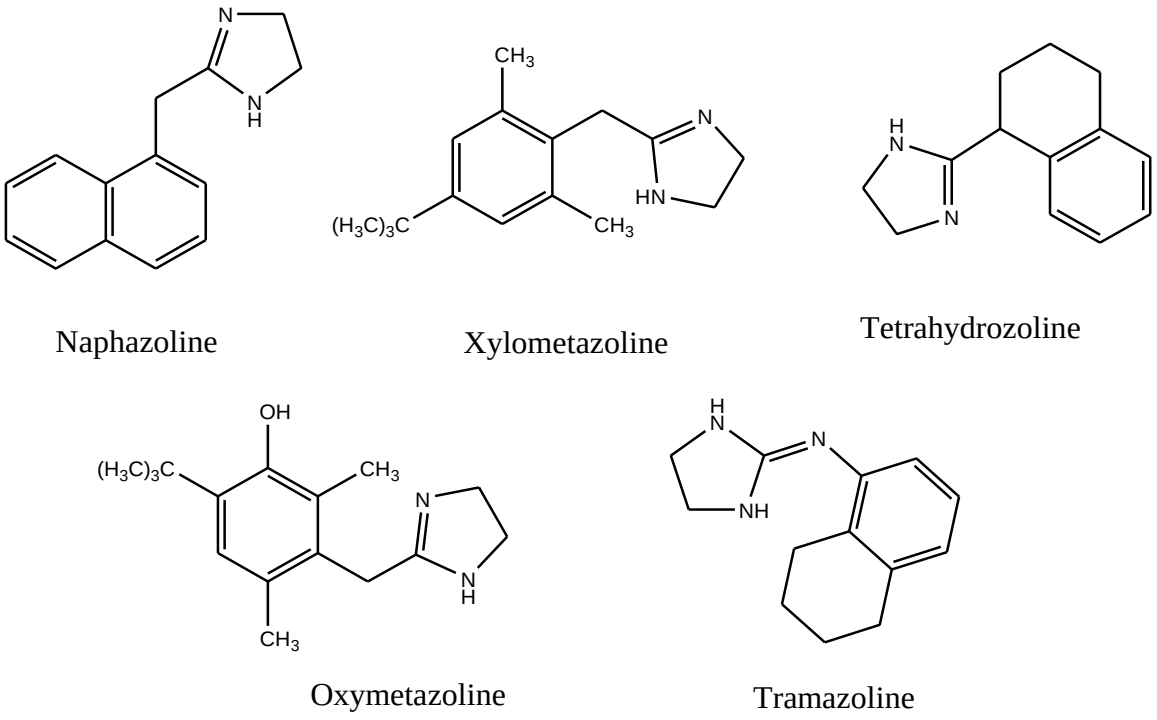


Figure 4. Chemical structure of alpha$_1$- agonist.

Xylometazoline is chemically [2-[(4-*tert*-butyl-2,6-dimethyl phenyl)methyl]4,5-dihydro-1*H*-imidazole]. It is a drug which is used as a topical nasal decongestant. Xylometazoline is an imidazole derivative which binds to α-ARs in the nasal mucosa.[46]

Because of its sympathomimetic effects, it should not be used in patients with high blood pressure, or other heart problems.

Oxymetazoline is chemically [3-(4,5-dihydro-1*H*-imidazole-2-yl-methyl)-2,4-dimethyl)-6-*tert*-butyl-phenol]. Oxymetazoline is a sympathomimetic that selectively agonises α_1 and partially α_2- ARs. The local vasoconstriction action of oxymetazoline results from their effect on endothelial postsynaptic α_2 receptors. Oxymetazoline is readily absorbed orally. [47]

Naphazoline is chemically [2-(1-naphtylmethyl)-2-imidazoline]. It is a sympathomimetic agent that acts as vasoconstrictor due to their effect on α- receptors in the arterioles of the conjunctiva, resulting in decreased congestion.

Tetrahydrozoline is chemically [(RS)-2-(1,2,3,4-tetrahydronaphthalen-1-yl)-4,5-dihydro-1*H*-imidazole], a derivative of imidazoline, an α- agonist and its main mechanism of action is the constriction of conjunctival blood vessels. This serves to relieve the redness of the eye caused by minor ocular irritants. [48]

Tramazoline is chemically N-(5,6,7,8-tetrahydronaphthalen-1-yl)-4,5-dihydro-1*H*-imidazole-2-amine. It is α-sympathomimetic agent, used locally in otorhinolaryngology.

Alpha$_2$-agonists are 2-aminoimidazoline centrally-acting vasodilators, used as antihypertensives, sedatives and for treatment of opiate dependence and alcohol withdrawal symptoms. [44, 45] Selected examples such as clonidine, guanabenz, guanfacine, brimonidine, tizanidine, apraclonidine are presented on Figure 5.

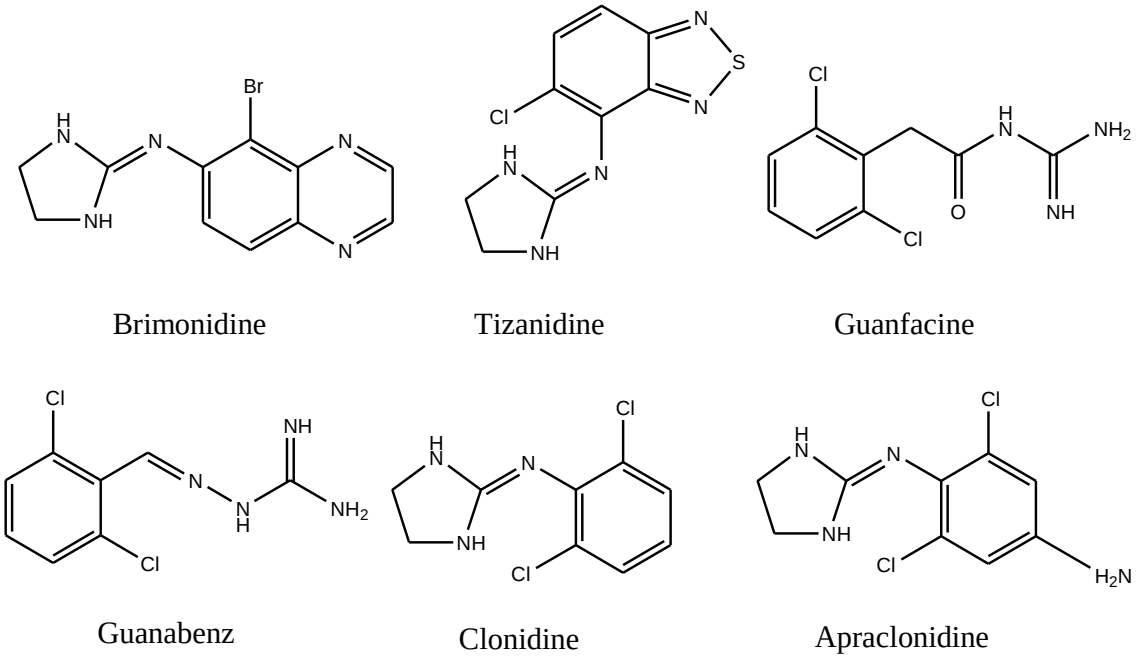


Figure 5. Chemical structure of alpha $_{2}$- agonists.

Clonidine is chemically [2-(2,6-dichlorophenylamino)-2-imidazoline]. It is an imidazoline derivative and centrally-acting α-adrenergic agonist, with antihypertensive activity. Clonidine bind to α_2-AR and decreased peripheral vascular resistance, decreased blood pressure, and decreased heart rate. In addition, clonidine binds to I_1- IR which may also contribute to a reduction in blood pressure. [49 -51] Chemically clonidine is characterized by presence of lipophilic *ortho*-dichloro substituent on the phenyl ring. The main difference between clonidine and α_1- agonist is the replacement of the carbon by an amine bridge. This make the imidazoline ring part of a guanidine group, and the uncharged form of clonidine exist as a pair of tautomers. [44, 45]

Apraclonidine is chemically [2,6-dichloro-N-(4,5-dihydro-1*H*-imidazol-2-yl) benzene -1,4-diamine] and does not cross the blood brain barrier which is correlated well with its log *P*, pK_a, and thus log D value. It is selective α_2- AR agonist and used specifically to control elevations in intraocular pressure by reduces production of aqueous humor and enhances outflow of aqueous humor, that can occur during laser surgery on the eye. Apraclonidine also has a neuroprotective effect through α_{2A} receptors located in the retina. [52]

Brimonidine is chemically [5-bromo-N-(4,5-dihydro-1*H*-imidazole-2-yl) quinoxalin-6 -amine]. Brimonidne can cross blood brain barrier and hence can produce hypotension and sedation effect. It is much more selective α_2- agonist than clonidine or apraclonidine and is used for treating glaucoma. [53]

Tizanidine is chemically [5-chloro-N-(4,5-dihydro-1*H*-imidazole-2-yl)benzo[c] [1,2,5] thiadiazol-4-amine], and used for treating spasticity associated with multiple sclerosis or spinal cord injury by stimulating α_2-AR. [54] Tizanidine is a potent sedative. It therefore has the potential to interact with other CNS depressants.

Guanabenz and Guanfacine are open-ring imidazoline. Guanabenz and guanfacine are structurally related compounds and have similar antihypertensive properties.

Guanfacine is chemically [N-(diaminomethylidene)-2-(2,6-dichlorophenyl) acetamide]. [44, 45]It is a sympatholytic drug used in the treatment of attention deficit hyperactivity disorder and hypertension. [55] It is a selective α_{2A} receptor agonist. [56] Guanfacine is more selective for α_2-AR than clonidine.

Guanabenz is chemically [2-(2,6-dichlorobenzylidene) hydrazine carboximidamide]. Guanabenz is an agonist of α_2-AR which is used as an antihypertensive drug. [57] Chemically, both drugs at physiological pH are exist in its non-ionized due to conjugation of the guanidine group with the bridging group which result in decrease the pK_a of the basic group, and this accounts for their CNS penetration and high oral bioavailability. [44, 45]

Imidazoline receptors

Recent research has identified the regulatory role of the IRs in sympathetic outflow and blood pressure regulation and this has led to the development of IR-ligands such as moxonidine and rilmenidine, which are currently used in treatment of high blood pressure and hyperglycemia. [58, 59] Their site of action is located in the central nervous system. IRs are receptors that recognize the imidazoline or oxazoline chemical structure. Activation leads to a centrally mediated hypotensive and anti-arrhythmogenic action. In recent, 3 subtypes of IRs have been identified: I_1-, I_2-, and I_3-IR. [60-62] The I_1-IRs are characterized by their high affinity to 2-aminoimidazolines ([^{3}H]-clonidine).

Imidazoline endogenous ligands

Imidazoline endogenous ligand (Clonidine Displacing Substance, CDS) was firstly identified in extracts of rat and bovine brain [63] but may also be present in peripheral tissue and in the circulation**.** [64, 65] Three different endogenous ligands have been characterized including agmatine (decarboxylated arginine) which binds with a moderate affinity to α_2-AR as well as to I_1- and I_2-IR. [66] β-carboline compounds, [67] such as harman [68] and harmalan [69], have been identified as putative endogenous substrates of either I_1- or I_2-IRs. These compounds (Figure 6.) have been shown that act as endogenous ligands at certain IRs and have central effects on blood pressure. [70]

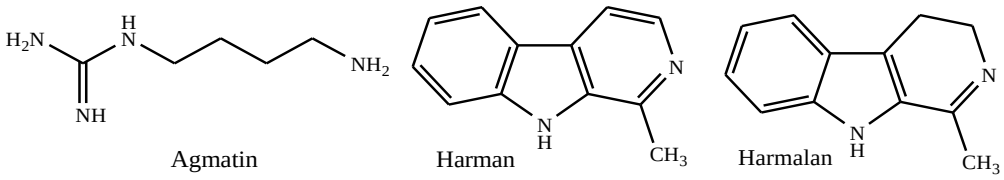


Figure 6. Chemical structure of imidazoline endogenous ligands.

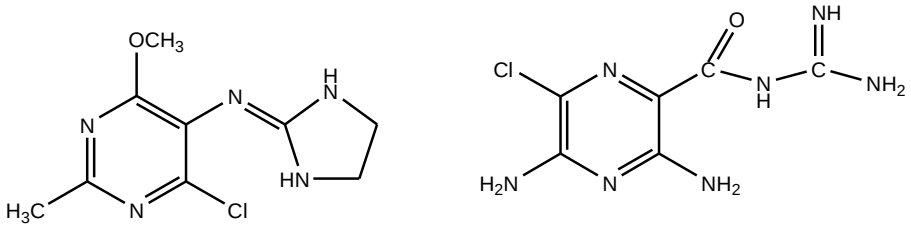
Agmatine [1-(4-aminobutyl) guanidine], is an aminoguanidine, which was first reported as putative endogenous IRs - ligand by Li et al in 1994. [66] Agmatine is aliphatic guanidine neurotransmitter found in the brain, with high affinity for IRs, I_1, I_2, α_2, and glutaminergic N-methyl-D-aspartate receptor channel (NMDA), where its bind as antagonist. It is biosynthesized by the decarboxylation of the amino acid, arginine, and metabolized by agmatase to the polyamine, putrescine, which bind to NMDA receptor channel. Agmatine stored in neurons and released in response to stress such as anxiety, depression and/or inflammation. The sympathoinhibition of agmatine includes α_2-AR and I_1-IR. [71] Although the physiologic role of agmatine in normal brain function is still unknown.

ß-carbolines such as harman [68] [1-methyl-9*H*-pyrido[3,4-b]indole] and harmalan [69] [1-methyl-3,4-dihydro-2*H*-pyrido[3,4-b]indole] and was recently identified as endogenous ligands that act at certain IRs and have central effect on blood pressure.[70] These compounds are formed from the condensation between indoleamins, such as

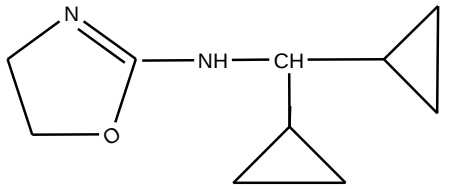
tryptamine, and short chain carboxylic acids (e.g, pyruvic acid) or aldehydes (e.g acetaldehyde). The potency of these compounds is similar to that of the clonidine. ß-carbolines can potentiate the rate of insulin secretion from human islets and suggest that they may be useful prototypes for the development of novel insulin secretagogues. [72]

Selective IRs-agonists

I1-IR agonists are drugs currently used in treatment of high blood pressure and hyperglycemia.[73] The mechanism by which central antihypertensives lowers blood pressure is due to activation of both α_2-AR and I_1-IRs. First-generation of centrally acting antihypertensive agents, such as imidazoline derivative clonidine, act mainly to stimulate these I_1-IR to lower blood pressure, but have sufficient agonism at α_2-ARs [49, 50] to produce side effects. The second-generation agents, such as rilmenidine and moxonidine, produce hypotension and sympathetic inhibition by an action principally on I_1-IRs rather than α_2-AR. [74-76] The low incidence of the side effects, antiarrhythmic effects, and beneficial metabolic and renal effects of second-generation of I_1-IR ligands suggest that they may provide a very useful antihypertensive therapy. [59] The selective I_1-IR agonists are capable of increasing the glucose-induced insulin secretion from pancreatic β-cells. [62, 77] Furthermore, moxonidine improves the metabolic profile in patients with hypertension and diabetes mellitus or impaired glucose tolerance.[78] The selective IR ligands are also more effective in regulation of body fat, neuroprotection, inflammation, cell proliferation, epilepsy, depression, stress, cell adhesion, and pain. Previously performed QSAR studies for the set of I_1-IR ligands indicated that an increase in lipophilicity, molar refractivity, and dipole moment value, together with a decrease in N-charge in the heterocyclic moiety effect on affinity for I_1-IR. Furthermore, highest occupied molecular orbital energy and lipophilicity of the ligands are important parameter for evaluation of I_1/α_2-selectivity. [20, 21]



Moxonidine Amiloride



Rilmenidine

Figure 7. Chemical structures of amiloride, moxonidine, and rilmenidine.

Amiloride [3,5-diamino-6-chloro-N-(diaminomethylene)pyrazine-2-carboxamide] is guanidinium derivatives, able to discriminate between I_2-IR subtypes, I_{2A}-IR and I_{2B}-IR. [79]

Rilmenidine [N-(dicyclopropylmethyl)-4,5-dihydro-1,3-oxazol-2-amine] is new generation of centrally acting antihypertensive agent. It is produce hypotension and sympathetic inhibition by an action principally on I_1-IRs rather than α_2-AR. The isosteric substitution of rilmenidine with a pyrrolinic ring blocked the binding affinity to α_2-AR, whereas I_1-IR affinity was hardly affected. [80]

Moxonidine is chemically [4-chloro-N-(imidazolin-2-ylidene)-6-methoxy-methylpyrimidine-5-amine]. Moxonidine is a new, second-generation centrally acting antihypertensive drug used for the treatment of mild to moderate essential hypertension. Moxonidine is an I_1-IR agonist that acts with minor activity at α_2-AR. Moxonidine also improved the metabolic profile of patient with hypertension and type two diabetic or impaired glucose tolerance. [78]

Chemical properties of moxonidine

From the aspect of chemical instability of the ligands of imidazoline and alpha adrenergic ligands especially interesting compound is pyrimidine derivative moxonidine, which easily undergoes to reaction of nucleophilic substitution. The official monographs in the European pharmacopoeia (EP) [81] and in the British pharmacopoeia (BP) [82] list four related substances of moxonidine; Impurity A (6-chloromoxonidine), B (4-methoxymoxonidine), C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine), of which impurity 6-chloromoxonidine and 4-methoxymoxonidine are process-related impurities which are related to the moxonidine and coming from the biosynthetic route itself while impurity 4-hydroxymoxonidine and 6-desmethylmoxonidine are degradants. The impurity types and ratios differ depending on the reaction condition. Position C4, C6, of pyrimidine ring is reactive to nucleophilic substituent. [83] Even a low level of humidity or the presence of other nucleophilic in the tablet matrix can affect the stability of moxonidine and generate impurities. According to EP [81] and BP [82] determination of moxonidine and its four related substances is performed by HPLC. Recently, the HPLC method [84] was developed and validated for the determination of moxonidine in the presence of its impurities, and the UPLC method [85] was devised as a stability indicating method for the determination of moxonidine and its degradation products in pharmaceuticals. Hydrophilic interaction liquid chromatography (HILIC) was devised for the separation of moxonidine in the presence of five related compounds as an alternative to the RP-HPLC for determination of polar analytes in a pharmaceutical matrix. [86] A stability indicating high performance thin-layer chromatographic method was reported and validated for analytical estimation of moxonidine in the presences of the degradants, excipients and impurities. [87] Several reports are available describing determination of moxonidine in human plasma by mean of liquid chromatography – electrospray ionization – mass spectrometry (LC-ESI-MS), [88] and gas chromatography-mass spectrometry (GC-MS). [89] The chemical structure of moxonidine and its four impurities are listed below in Figure 8.

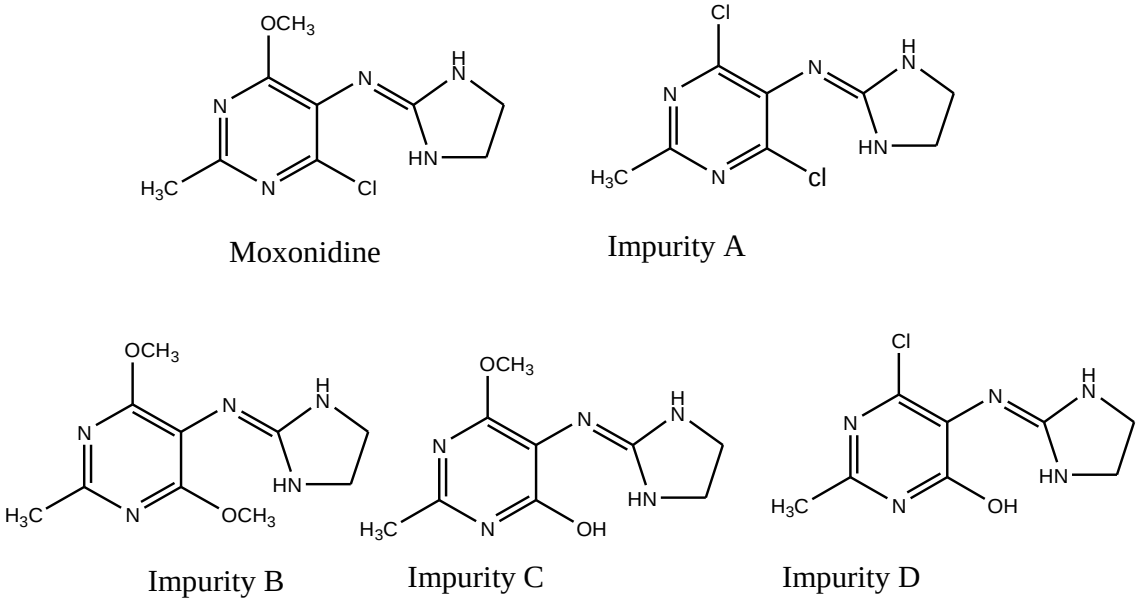


Figure 8. Chemical structure of moxonidine and its impurities.

Synthesis of moxonidine

Moxonidine is obtained directly from the reaction of 4,6-dichloro-2-methyl-5-(1-acyl-2 -imidazolin-2-yl)-amino pyrimidin (DMAIA) with sodium methoxide CH_3ONa in the presence of methanol CH_3OH. [90] The moxonidine precursor DMAIA was obtained by coupling of 5-amino-2-methyl-4,6-dichloro-pyrimidine with 1-acetylimidazolidin-2- one in the presence of phosphoryl chloride. 5-amino-2-methyl-4,6-dichloro-pyrimidine was prepared in several steps from 4,6-dihydroxy-2-methylpirimidine after nitration to 4,6-dihydroxy-2-methyl-5-nitropirimidine and reduction to 5-amino-4,6-dihydroxy-2-methylpirimidine. The final reaction is replacement of the hydroxyl groups of last intermediates by chlorine atoms and obtaining 5-amino-2-methyl-4,6-dichloro-pyrimidine. On exposure to humidity and high temperature degradation of moxonidine can occur. The products of degradation of moxonidine are known as impurities A (6-chloromoxonidine), B (4-methoxymoxonidine), C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine). The impurities type and their ratio depend on the reaction conditions. Impurity A (6–chloromoxonidine) can appear as a product of side reaction DMAIA with sodium hydroxide at 50^0 C for 4 hours. Impurity B (4–methoxymoxonidine)

can appear either as a product of side reaction of DMAIA with sodium methoxide in DMF at 120^0 C for four hours or as an degradant of moxonidine after heating with sodium methoxide under similar condition. Impurity C (4– hydroxymoxonidine) can arise either as degradant of impurity D (6– desmethylmoxonidine) after heating in the presence of sodium methoxide in methanol or can arise as a product of side reaction of moxonidine under harsher conditions. Impurity D (6–desmethylmoxonidine) can appear as degradant of impurity B (4– methoxymoxonidine) after the cleavage of ether bond (Figure 9.). [83]

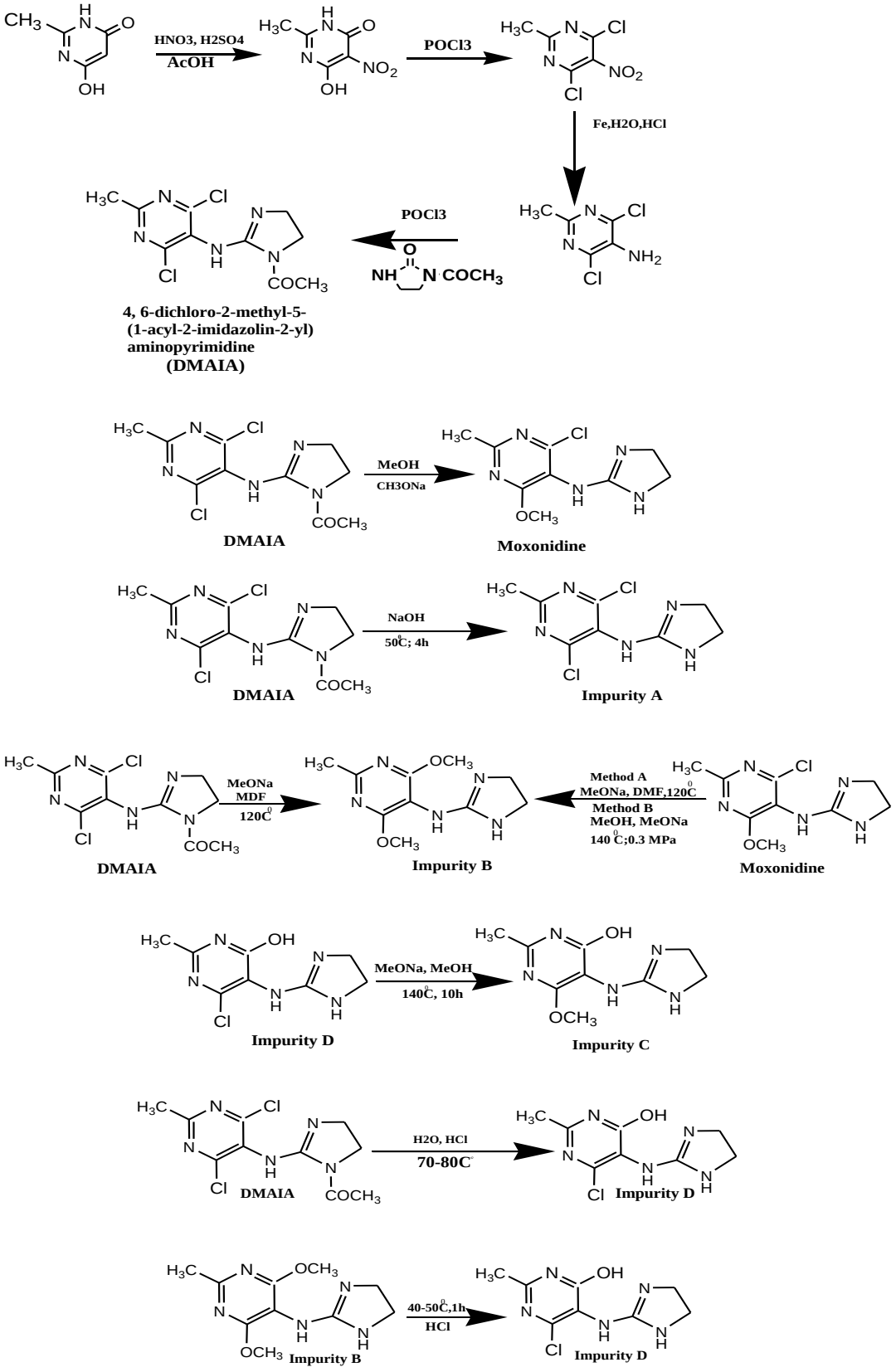


Figure 9. Synthesis of moxonidine and its impurities.

Theoretical study has been performed on physicochemical properties of a series of structurally similar drugs acting on the I_1 and α_2-AR [91] and the following conclusion was obtained:

- Moxonidine prefer a structure with an exocyclic double bond (iminoform)
- One of the characteristics of moxonidine was the non existance of any conjugation electronic interaction between pyrimidine and imidazoline ring. The computed stable conformation for moxonidine species is characterized by the pyrimidine and imidazoline rings being in the mutual gauch conformation. This conformation is stabilized by the favorable intra-molecular hydrogen bond between the N-H group of the imidazoline group and the oxygen atom of the methoxy group of the pyrimidin part of drug.
- The primary protonation site is imidazoline part of moxonidine and moxonidine is practically an equally basic drug to its parent clonidine.
- The moxonidine base was found to be less substantially lipophilic than the base of clonidine.

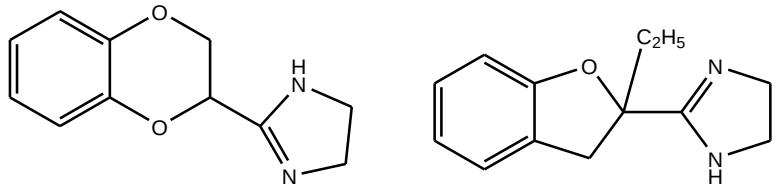
One of the most important characteristic of centrally acting antihypertensive drugs is their different selectivity by blockade of α_2-AR and IRs. The substantially higher affinity of moxonidine toward I_1-IRs in comparison with clonidine could be explained by its lower lipophilicity and the different spatial arrangement of the biologically active conformation. Moxonidine and clonidine aromatic groups adopt a different conformation. It is probable that the spatial arrangement of moxonidine fits better the physicochemical nature of the I_1-IR involved in ligand binding.

IRs-antagonists

Efaroxan [2-(2-ethyl-2,3-dihydro-1-benzofuran-2-yl)-4,5-dihydro-1*H*-imidazole] is a potent, highly selective α_2-AR antagonist and I_1-IR antagonist and is a selective agonist at the I_3- receptor. It blocks ATP- sensitive potassium channels in pancreatic β cells and induces insulin release. [92, 93]

Idazoxan is [2-[2-(1,4-benzodioxanyl)]-2-imidazoline]. It is the most selective α_2-AR blocking agent and an antagonist for the IR. [94] Idazoxan is the I_2-IR prototype ligand. At peripheral sites, idazoxan antagonized the effects of α_2- agonists such as clonidine but was

ineffective against α_1- agonists such as cirazoline and phenylephrine. Idazoxan is the most potent selective α_2-AR that are used for the investigation of peripheral and central α_2-ARs (Figure 10.).



Idazoxan Efaroxane

Figure 10. Chemical structures of idazoxan and efaroxan.

1.5. Quantitative structure retention relationship of imidazoline and alpha adrenergic receptors ligands

Chromatographic retention prediction methodologies are classified into two main categories: In the first category, prediction model is built to describe the retention of investigated compounds under changing chromatographic condition and is applied in computer-assisted method. It is usually used during chromatographic method development and based on the linear solvent strength. In the second category, prediction model is formed by Linear Solvation Energy Relationship (LSER) and by Quantitative Structure Retention Relationship (QSRR). In LSER, the experimental molecular descriptors obtained from solvatochromic measurement are usually used to describe retention in reversed-phase liquid chromatography (RPLC), normal-phase liquid chromatography (NPLC), whereas in QSRR, the theoretical molecular descriptors that are generated by calculation chemistry are usually used to describe the chromatographic retention of investigated compounds. The aim of both methodologies is to derive a model to describe the chromatographic retention on a given chromatographic system, which then can be used for retention prediction of new compounds. The difference between two models (LSER, QSRRs), is the nature of the molecular descriptors used in the model. [95] As noted, QSRR is useful technique that shows relationship between chromatographic

properties and molecular descriptors characterizing the structure of investigated compounds. [96, 97] In the QSRR study regarding TLC, correlation can be established between retention data (mostly R_M^0 value) and various empirical and non-empirical structural parameters. [26, 98, 99] Different statistical methods such as Multiple Linear Regression (MLR), Artificial Neural Network (ANN) and Partial Least Squares Regression (PLS) can be used for QSRR model building. [26, 97, 100, 101] In addition, Principle Component Analysis (PCA) is very useful tool in providing data overview. [98, 102] As a result of statistical modelling, established QSRR models can be applied for identification of the most useful structural descriptors, prediction of the retention for new synthesized molecules and identification of unknown analytes. [28, 103] QSRR can provide insight into the molecular mechanism of separation in a selected chromatographic system and quantitative comparison of separation properties of individual types of chromatographic conditions. Literature overview showed that QSRR of selected imidazoline derivatives was performed on α_1-acid glycoprotein column. [100] In addition, the retention behavior of imidazoline and alpha adrenergic ligands has been investigated using the HPLC system [104- 108] and TLC system. [26]

1.5.1. Molecular descriptors used in developing QSRR models

Molecular descriptors are numerical values that characterized properties of molecules e.g physico-chemical properties. There are several ways of classification of molecular descriptors. One of the simplest is based on the nature of descriptor on *experimental* molecular descriptors and *theoretical* molecular descriptors. Experimental molecular descriptors (physico - chemical properties) are results of the standard experiment. They are generally related to the retention, but they are often either unavailable or contain large of errors. Theoretical molecular descriptors are derived from a symbolic properties of the molecule and are easily calculated, but they are not always related to the specific retention phenomena. [103] Theoretical descriptors can be further classified into:

- Zero-dimensional (0D) descriptors- It's the most simplest descriptors, independent of the molecular structure and derived from molecular formula (molecular weight, number of atoms etc.)

- One-dimensional (1D) descriptors-represent molecule considering its functional groups (i.e. list of structural fragments, charge descriptors)
- Two-dimensional (2D) descriptors-take into account topological representation of the molecule, connection between atoms and type of bonding (Balaban index, Zagreb index)
- Three-dimensional (3D) descriptors-are calculated from the three-dimensional representation of the molecule (3D-MoRSE descriptors, WHIM descriptors, GETAWAY descriptors)
- Four-dimensional (4D) descriptors are calculated from the lattice or stereoelectronic representation of the molecule.

Molecular descriptors can be also classified into physico-chemical, theoretical, quantum chemical. Quantum chemical descriptors provide insights into the mechanism of chromatographic retention at the molecular or even sub-molecular level. However, their correlation with retention is rather weak and they also are not easy to calculate. [109]

1.5.2. Chemometric methods in structure-retention relationship analysis

Partial least square regression

PLS is regression method that is used to correlate the relationship between two data matrices, **X** (latent variables or independent variables) and Y (dependent variables), by a linear multivariate model. It was recently developed by the Swedish statistician Herman Wold in 1975. PLSR is more convenient than MLR, because of its ability to analyze data with many, noisy, collinear, and even incomplete variables in both **X** and **Y** matrices. Also, by PLS method models with high predictive power can be obtained which enable creation of reliable QSAR and QSRR models regardless of the number of examined compounds and molecular descriptors. In addition, the precision of PLS model parameters can be further improved with the increasing number of relevant variables and observations. [110]

Principal component analysis

The Principal component analysis is one of the most common chemometric methods used in a two-way data analysis. In PCA, data are organized in two-way matrix, X (m × n), where **m** and **n** denote, respectively, the number of objects and the number of examined

variables. PCA is one of the multivariate methods that provides an opportunity for investigation of the existence of regularities in large data set, providing thus information about compounds which behave in a similar way. In addition, by analyzing data matrix another goals could be also achieved, such as: simplification, data reduction, outlier detection, variable selection, classification, etc. [111]

1.5.3. Model validation

Validation of the developed mathematical models is an important aspect of any QSRR study. Once a model is obtained, it is necessary to determine its reliability and statistical significance by use of the cross-validation and external test set prediction.

For validation of QSRR models, usually various strategies are adopted:

1. Internal validation or cross-validation (actually, while extracting data, cross validation is a measure of model robustness, the more a model is robust (higher Q^2) the less data extraction perturb the original model). 2. External validation by splitting the available data set into training set for model development and prediction set for model predictivity evaluation. 3. Blind external validation by application of model on new external data.

4. Data randomization or Y-scrambling for verifying the absence of chance correlation between the response and the modeling descriptors. [112, 113]

2. Objective

The main objectives of this work were:

- Investigation of chromatographic behaviour and lipophilicity of alpha adrenergic and imidazolin receptor ligands (clonidine, moxonidine, guanfacine, brimonidine, efaroxan, idazoxan, harmane, harmine, tizanidine, naphazoline, xylometazoline, tetrahydrozoline, oxymetazoline, tramazoline, and amiloride) by reverse phase-thin layer chromatography using different stationary and mobile phases and different mode of development (horizontal and vertical)

- Development of QSRR models enabling better understanding of retention mechanism in different mobile phase/stationary phase TLC systems and prediction of the retention behavior of the related guanidine and imidazoline derivative.

- Development and validation of a novel, reproducible, simple, sensitive, accurate and precise thin layer chromatographic method for the analysis of moxonidine and its impurities in pharmaceuticals dosage form.

3. Experimental Part

3.1. Examination of retention behaviour and lipophilicity of imidazoline and alpha adrenergic receptors ligands

3.1.1. Apparatus and reagents

- TLC plates: octadecyl silica plates (the TLC Silica gel 60 RP-18 F_{254s} pre-coated aluminium sheets, Merck, Darmstadt, Germany) and CN-modified silica plates (the HPTLC Silica gel 60 CN F_{254s}, Merck, Darmstadt, Germany)
- Nanomat III (Camag, Muttenz, Switzerland), manual sample applicator
- Camag twin trough chamber 10x10 cm (Camag, Muttenz, Switzerland)
- Horizontal chamber 10x10 cm (Camag, Muttenz, Switzerland)
- Syringe 10 μL (Hamilton, Bonaduz, Switzerland)
- Camag UV lamp dual wavelength (Camag, Muttenz, Switzerland)
- Methanol (Merck, Darmstadt, Germany), tetrahydrofuran (Sigma-Aldrich, St. Louis, MO, USA), ammonium hydroxide (Carlo Erba, Milan, Italy) and water used to prepare mobile phases were of the analytical purity grade.
- The following standards: clonidine hydrochloride, moxonidine hydrochloride, guanfacine hydrochloride, brimonidine tartrate, efaroxan hydrochloride, idazoxan hydrochloride, harmane, harmine hydrochloride, tizanidine hydrochloride, naphazoline hydrochloride, xylometazoline hydrochloride, tetrahydrozoline hydrochloride, oxymetazoline hydrochloride were purchased from Sigma-Aldrich, St. Louis, MO, USA; tramazoline hydrochloride and amiloride hydrochloride were obtained from Zdravlje-Actavis, Leskovac, Serbia and Galenika, Belgrade, Serbia, respectively.

3.1.2. Sample preparation

The investigated compounds were dissolved in methanol (2 mg mL^{-1}) and the 3 μL aliquots of each solute were spotted onto the plates.

3.1.3. Chromatography

Chromatographic behavior of tested compounds was examined in three different mobile phase/stationary phase systems and their composition is presented in Table 2. In the applied chromatographic systems the content of tetrahydrofuran and methanol was changed in the 5% steps, while the content of ammonia was kept constant at 5 vol%.

Table 2. Examined chromatographic systems

Stationary phase	Mobile phase	Volume fraction of organic modifier in the mobile phase
RP-18	methanol-water	0.55, 0.60, 0.65, 0.70, 0.75, 0.80, 0.85
RP-18	tetrahydrofuran-ammonia-water	0.60, 0.65, 0.70, 0.75, 0.80
CN	tetrahydrofuran-ammonia-water	0.55, 0.60, 0.65, 0.70, 0.75
RP-18 (horizontal development)	tetrahydrofuran-ammonia-water	0.60, 0.65, 0.70, 0.75, 0.80

The plates were developed in the ascending and horizontal modes, without pre-saturation of the chromatographic chamber with the methanol-water mobile phase and after 15 min pre-saturation with the tetrahydrofuran-ammonia-water mobile phase. Zone detection was performed in the UV light at 254 nm. The retardation factor (R_f values) were calculated as an average from the three chromatograms:

$$R_f = a/b \qquad (7)$$

Where **a** is distance from the start to the center of the zone corresponding to examined compound and **b** is distance from the start to the front of mobile phase.

TheR_f values obtained in the three different chromatographic systems, i.e., methanol–water/RP-18, tetrahydrofuran-ammonia-water/RP-18 and tetrahydrofuran-ammonia-water/CN were further used for calculation of the retention parameter R_M, according to the Bate–Smith and Westall equitation (4). [30]

For each compound and each chromatographic system, retention parameter R_M^0 corresponding to pure water was obtained as an extrapolated value to 0% organic modifier in the mobile phase, using linear equitation (5).

Parameter C_0 was calculated according to equitation (6) and along with R_M^0 and *m* were used as experimental lipophilicity indices for evaluation of lipophilicity of imidazoline and alpha adrenergic receptors ligands.

3.1.4. Calculation of log *P* values

The lipophilicity parameter (log *P*) was computed using several softwares, i..e., Virtual Computational Chemistry Laboratory (Alog *Ps*, AClog *P*, Alog *P*, Mlog *P*, KOWWINlog *P*, Xlog *P2*, Xlog *P3*), [114] Molinspiration Cheminformatics (milog *P*), [115] Marvin 5.5.1.0 ChemAxon (ChemAxon log *P*), [116] (ACD/Labs) Software (ACD/log *P*) [117] and CS Chem Office, version 7.0 (Clog *P*).[118]

3.2. QSRR study of imidazoline and alpha adrenergic receptors ligands

3.2.1. Geometry Optimization

Among the analyzed compounds, 2-aminoimidazolines such, as moxonidine, clonidine, tizanidine, brimonidine and tramazoline can exist in two major tautomeric forms, amino and imino. Using the B3LYP/6–31G (d, p) level of the Density Functional Theory (DFT) [119] neutral forms of the respective amino and imino tautomers were optimized in the Gaussian 98 program. [120] The selected basis set proved to be a good choice for the examination of the related amidines and guanidines.[91] Based on the obtained Self Consistent Field Energy, the respective imino tautomers were selected as the more stable forms and then used for further calculations.

Calculation of pK_a and selection of a predominant molecular/cationic/anionic form of the analyzed compounds at a given pH value of an aqueous phase, was performed using the Marvin 5.5.1.0 ChemAxon program. [116] The geometries of the examined ligands were fully optimized at the B3LYP/3–21(d, p) levels of the DFT in the Gaussian 98 program. [120]

3.2.2. Calculation of molecular descriptors

The Gaussian 98 (B3LYP/3-21 G(d,p) basis set), [120] the Marvin 5.5.1.0 ChemAxon, [116] the Chem3D Ultra 7.0.0, [121] the Molinspiration Cheminformatics, [115] and the Dragon[122] programs were applied for the computation of physicochemical, constitutional, thermodynamic and electronic properties of the analyzed compounds.

Molecular descriptors calculated in the Marvin 5.5.1.0 ChemAxon, Chem3D Ultra 7.0.0 and Gaussian 98 programs are presented in Table 3.

Table 3. Molecular descriptors calculated in the Marvin 5.5.1.0 ChemAxon, Chem3D Ultra 7.0.0 and Gaussian 98 programs

Programs	Molecular descriptors
Marvin 5.5.1.0 ChemAxon	Polarizability, Polar Surface Area, Molecular Surface Area, Van der Waals surface area, Refractivity, H bond donor, H bond akceptor, Charge, Partition coefficient, Distribution coefficient.
Chem3D Ultra	Connolly Accessible Area, Connolly Molecular Area, Connolly Solvent-Excluded Volume, Molecular Weight, Ovality, Principal Moment of Inertia - X, Principal Moment of Inertia - Y, Principal Moment of Inertia - Z, Molar Refractivity, Partition Coefficient (Octanol/Water), Non-1,4 VDW Energy, VDW 1,4 Energy, Balaban Index, Cluster Count, Shape Attribute, Shape Coefficient, Wiener index.
Gaussian	Highest Occupied Molecular Orbital (HOMO), Highest Unoccupied Molecular Orbital (LUMO), Dipole.

Also, the quantum chemically-based reactivity descriptors (such as chemical potential (μ), electronegativity (χ), hardness (η), global softness (S), and electrophilicity index (ω)) were added to the set of the calculated properties and were calculated according to the following equations: [123]

$$\mu = \frac{(E_{LUMO} + E_{HOMO})}{2} \qquad (8)$$

$$\chi = -\mu \qquad (9)$$

$$\eta = \frac{(E_{LUMO} - E_{HOMO})}{2} \qquad (10)$$

$$S = \frac{1}{2\eta} = \frac{1}{(E_{LUMO} - E_{HOMO})} \qquad (11)$$

Where E_{LUMO} is energy of the lowest unoccupied molecular orbital and E_{HOMO} is energy of the highest occupied molecular orbital.

3.2.3. Partial least squares modeling

The Soft Independent Modeling of Class Analogy SIMCA P+ 12.0 program [124] was used for thePLS analysis and the QSRR modeling. The experimentally obtained R_M^0 values and the computed molecular descriptors of the examined compounds were used for the QSRR analysis. The PLS methodology was applied for the calculation of Variable Importance in the Projection (VIP) and for building of the QSRR models. A summary of the importance of each variable (X_k) for both, Y and X matrices, is given as the VIP_k parameter. Among all molecular properties that were included in generating the QSRR models, only the variables with the VIP values higher than 0.5 have been considered for the regression. In the course of building the models, molecular properties with the lowest VIP values were successively removed from the PLS models, until the best model was obtained. Optimal combinations of the most significant descriptors for building of the QSRR models were selected by the squares of the multiple correlation coefficients R^2, Q^2 (a cross-validated correlation coefficient), the root mean square error of estimation (RMSEE) for the training set, and the root mean square error of prediction (RMSEP) for the test set. Models with $Q^2 \geq 0.5$ can be considered as those having a good predictive capability. [125]

$$Q^2 = 1 - \frac{PRESS}{\sum (Y_{obs(training)} - \overline{Y}_{training})^2} \qquad (12)$$

$$PRESS = \sum_{i=1}^{n} e_{(i)}^2 \qquad (13)$$

$$RMSEE = \sqrt{\frac{\sum_{i=1}^{n} (Y_{obs(training)} - Y_{pred(training)})^2}{n}} \qquad (14)$$

In the above equations PRESS is parameter calculated for the training set according to Eq. (13) using leave-one out cross-validation (LOO-CV) approach, where $e_{(i)}^2$ is the squared sum of differences between observed and LOO-predicted values. In LOO-CV

approach each compound of the training set was deleted once and a new model was built with the remaining compounds and used to predict the Y-value of deleted compound. Procedure was repeated until all compounds have been deleted once. In the Eq. (12) and (14) $Y_{obs(training)}$ is an observed R_M^0 value of a compound in the training set, $\overline{Y}_{training}$ is average R_M^0 values of compounds from the training set, whereas $Y_{pred(training)}$ is predicted R_M^0 values of compounds from the training set, and *n* is the number of compounds in the training set.

Apart from LOO-CV method, the examined data set of 16 compounds was divided into the training set consisting of 12 compounds (moxonidine, brimonidine, clonidine, amiloride, guanabenz, idazoxan, efaroxan, harmane, naphazoline, tramazoline, oxymetazoline, and xylometazoline), used for building of the models, and the test set consisting of 4 compounds (tizanidine, guanfacine, harmine, and tetrahydrozoline), used for an external validation. The same training and test sets were used for the formation of all QSRR models. Predictive power of all created models was estimated by external validation using following parameters in Eq. (15)

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{n} (Y_{obs(test)} - Y_{pred(test)})^2}{n}} \qquad (15)$$

Where RMSEP is root mean squared error of prediction, *n* is the number of compounds in the test set, while $Y_{obs(test)}$ and $Y_{pred(test)}$ represent experimental and predicted values for compounds in the test set.

The response permutation test (Y scrambling), as a measure of the model over fitting, was used to examine the over fitting due to the chance correlation and the statistical significance of the R^2 and Q^2 values. [125] In this test, the Y-variables were randomly re-ordered 100 times whereas the X-matrix was left intact. Model was fitted to permuted data and the new R^2 and Q^2 parameters were calculated. All model selection steps were repeated on the scrambled Y response data. Regression lines were fitted through the R^2 and Q^2 values in order to obtain two separate intercepts. The intercepts of these regression lines should not be higher than 0.3–0.4 for R^2-intercept and 0.05 for Q^2-intercept. [125]

3.3. Quantitative analysis

3.3.1. Apparatus and reagents

- Chromatographic plates pre-coated with silica gel 60 F_{254} (Merck, Darmstadt, Germany)
- Camag twin trough chamber 20 ×10 cm (Camag, Muttenz, Switzerland)
- Camag Linomat 5 (Camag, Muttenz, Switzerland)
- Camag TLC scanner II (Camag, Muttenz, Switzerland)
- Ultrasonic bath UCI-75 (R. ESPINAR, S.L., Barcelona, Spain)
- Standard substances :

  -*Moxonidine*: 4-chloro-N-(imidazolidine-2-ylidene)-6-methoxy-2-methylpyrimidin-5- amine

  -*Impurity A*: 4,6-dichloro-N-(imidazolidine-2-ylidene)-2-methylpyrimidin-5-amine (6-chloromoxonidine)

  -*Impurity B*: N-(imidazolidin-2ylidene)-4,6-dimethoxy-2-methyl-pyrimidin-5-amine(4-methoxymoxonidine)

  -*Impurity C*: 5-[(imidazolidine-2-ylidene)amino]-6-methoxy-2-methylpyrimidin-4-ol (4-hydroxymoxonidine)

  -*Impurity D*: 6-chloro-5-[(imidazolidine-2-ylidene)amino]-2-methylpyrimidin-4-ol were obtained by (Chemagis, BneiBrak, Israel).
- The Moxogama® 0.4 mg film tablets were manufactured by Worwag Pharma (Boblingen, Germany).
- Placebo mixture

  -Lactose monohydrate (Zorka Pharma, Serbia)

  -Povidone K-25 (Sigma-Aldrich Chemie GmbH, Germany)

  -Crospovidone (B.M.P. Bulk Medicines & Pharmaceuticals GmbH, Germany)

  -Magnesium stearate (Merck, Germany)
- Solvents used for preparation of mobile phase

  -Toluene (POCH, Gliwice, Poland)

  -1, 2 dichloroethane (Fisher chemical, Loughborough, UK)

  -Ammonia solution 25% (Merck, Darmstadt, Germany)

  -Methanol (Merck, Darmstadt, Germany)

3.3.2. Experimental design

The experimental scheme was obtained by reduced central composite face-centered design using Modde® software. Based on the observation obtained during the preliminary studies four factors, *i.e*. methanol content in the total mobile phase (x_1), saturation time (x_2), band width (x_3) and developing distance (x_4) were selected for screening. The SIMCA P+ 12.0 program [124] was used for investigation of the influence of the examined factors on the resolution between impurities C (4-hydroxymoxonidine) and D (6-desmethylmoxonidine) (Rs(C/D)), and moxonidine and impurity A (6-chloromoxonidine) (Rs (M/A)) calculated according following equation:

$$Rs = 1.18\ a\ ((R_{f2} - R_{f1})/(w_{h1} + w_{h2}) \qquad (16)$$

Where R_{f2} and R_{f1} represent ratios of the distances from the point of application to the centers of the spots and the distance travelled by the solvent front from the point of application; w_{h1} and w_{h2} are peak widths at half-height, and a is migration distance of the solvent front. [81]

Examined factors and their interactions were used as independent variables X ($x_1, x_2, x_3, x_1 \times x_1, x_2 \times x_2, x_3 \times x_3, x_1 \times x_2, x_1 \times x_3, x_2 \times x_3$) while resolutions ((Rs(C/D), Rs(M/A)) were used as independent variables during the PLS modeling.

3.3.3. Preparation of solutions for validation of TLC method

3.3.3.1. Preparation of stock solutions

Stock solution of moxonidine

Quantity equal to 25 mg of moxonidine is accurately weighed, transferred into a volumetric flask of 25 mL and dissolved in 15 mL of methanol using an ultrasonic bath. Solution is filled with methanol to the mark. The concentration of solution is 1 mg/mL.

Stock solution of impurities

Quantity equal to 5 mg of each impurity (A 6-chloromoxonidine, B 4-methoxymoxonidine, C 4-hydroxymoxonidine, and D 6-desmethylmoxonidine) is accurately weighed, transferred into a volumetric flask of 25 mL and dissolved in 15 mL of methanol using an ultrasonic bath. Solution is filled with methanol to the mark. The concentration of each impurity in the solution is 0.2 mg/mL.

3.3.3.2. Preparation of standard solutions

Preparation of standard solutions for assessment of the method linearity

Volumes equal to 2 mL, 3 mL, 4 mL, 5 mL and 6 mL of stock solution of moxonidine are transferred into volumetric flasks of 10 mL and filled with methanol to the mark. The final concentration of moxonidine are 0.2 mg/mL, 0.3 mg/mL, 0.4 mg/mL, 0.5 mg/mL, and 0.6 mg/mL, respectively. The 1 µl of each solution is applied in triplicate to the chromatographic plate (corresponding to 200 ng, 300 ng, 400 ng, 500 ng, and 600 ng, respectively per band) band-wise with the 10 mm band width, with an application rate of 100 nL s^{-1}. Volume equal to 1.0 mL, 2.0 mL, 3.0 mL, 5.0 mL and 8.0 mL of stock solution of impurities are transferred into volumetric flasks of 10 mL and filled with methanol to the mark. The obtained concentrations of each impurity are 0.02 mg/mL, 0.04 mg/mL, 0.06 mg/mL, 0.1 mg/mL, 0.16 mg/mL, respectively. The 1 µl of each solution, including stock solution of impurities is applied in triplicate to the chromatographic plate (corresponding to 20 ng, 40 ng, 60 ng, 100 ng, 160 ng, and 200 ng, respectively per bend) band-wise with the 10 mm band width, with an application rate of 100 nL s^{-1}

Preparation of standard solutions for assessment of the method precision

Solution of moxonidine for assessment of the method precision

Volumes equal to 2 mL, 4 mL, and 6 mL of stock solution of moxonidine are transferred into volumetric flasks of 10 mL and filled with methanol to the mark. The final concentrations of moxonidine are 0.2 mg/mL, 0.4 mg/mL, and 0.6 mg/mL, respectively. The 1 µl of each solution is applied 6 times to the chromatographic plate (corresponding to 200 ng, 400 ng, and 600 ng, respectively per band).

Solutions of impurities for assessment of the method precision

Volumes equal to 2.5 mL, 4 mL, and 9.5 mL of stock solution of impurities are transferred into volumetric flasks of 10 mL and filled with methanol to the mark. The obtained concentrations of each impurity are 0.05 mg/mL, 0.08 mg/mL, and 0.19 mg/mL, respectively. The 1 µl of each solution is applied 6 times to the chromatographic plate (corresponding to 50 ng, 80 ng, and 190 ng, per band).

Preparation of standard solutions for assessment of the method accuracy

Standard solution of moxonidine for assessment of the method accuracy

Volumes equal to 1.6 mL, 2.0 mL, and 2.4 mL of stock solution of moxonidine are transferred into volumetric flasks of 5mL and filled with methanol to the mark. The obtained concentrations of moxonidine are 0.32 mg/mL, 0.4 mg/mL, and 0.48 mg/mL, respectively.

Standard solution of impurities for assessment of the method accuracy

Volumes equal to 1.25 mL of stock solution of impurities are transferred into volumetric flasks of 25 mL and filled with methanol to the mark. The obtained concentration of each impurity in the mixture is 0.01 mg/mL.

Working standard solution of impurities for assessment of the method accuracy

Volumes equal to 0.6 mL, 1.0 mL, and 2.4 mL of standard solution of impurities for assessment of the method accuracy are transferred into volumetric flasks of 5 mL and filled with methanol to the mark. The obtained concentrations are: 0.0012 mg/mL, 0.002 mg/mL and 0.0048 mg/mL, respectively.

Preparation of solution of laboratory mixture for assessment of the method accuracy

Preparation of laboratory mixture (placebo mixture)

Quantities equal to 4.8 g of lactose monohydrate, 0.1 g of povidone K-25, 0.25 g crospovidone and 0.015 g of magnesium stearate are transferred in mortar and mixed until homogenous mixture is obtained.

Solutions of moxonidine for assessment of the method accuracy

Quantity equal to 0.498 g of placebo mixture (corresponding to mass of tablets which contain 2 mg of moxonidine) are transferred in three volumetric flasks of 5 mL. Volumes equal to 1.6 mL, 2.0 mL, and 2.4 mL of stock solution of moxonidine are added to measured placebo mixture and sonicated in 4 mL of methanol for 20 min using an ultrasonic bath. Solutions are filled up to 5 mL with same solvent, and then centrifuged at 3000U/min for 15 min. The obtained supernatants are filtered through the 0.45 μm pore size membrane filters (Millipore). The final concentrations of moxonidine are 0.32 mg/mL, 0.4 mg/mL and 0.48 mg/mL, respectively. The 1 μl aliquot of the filtrates is applied to the chromatographic plates by Linomat 5 (corresponding to levels of 80%, 100%, and 120%, respectively).

Solutions of impurities for assessment of the method accuracy

Quantities equal to 0.498 g of placebo mixture (corresponding to mass of tablets which contain 2 mg of moxonidine) are transferred in three volumetric flasks of 5 mL. Quantities equal to 2 mg of moxonidine working standard and volumes equal to 0.6 mL, 1.0 mL and 2.4 mL of stock solution of impurities for assessment of the method accuracy are added to measured placebo mixture and sonicated in 4 mL of methanol for 20 min, using an ultrasonic bath. Solutions are filled up to 5 mL with methanol, and then centrifuged at 3000U/min for 15 min. The obtained supernatants are filtered through the 0.45 μm pore size membrane filters (Millipore). The obtained concentrations of impurities are 0.0012 mg/mL, 0.0020 mg/mL and 0.0048 mg/mL, respectively. The 40 μl aliquot of the filtrates is applied to the chromatographic plates in the form of band with 10 mm width, and an application rate of 100 n L s^{-1}. (corresponding to levels of 0.3%, 0.5%, and 1.2%, respectively)

3.3.3.3. Preparation of sample solution

Ten tablets from which the film was previously removed are weighed and pulverized. The quantity of the powdered tablets equivalent to 2.0 mg of moxonidine is transferred to the 5 mL volumetric flask and sonicated in 4 mL methanol for 20 min using an ultrasonic bath. The solution is made up to 5 mL with the same solvent, and then centrifuged at 3000U/min for 15 min. The obtained supernatant is filtered through the 0.45 mm pore size membrane filter (Millipore). For an assay of moxonidine and impurities, the 1 μl and 40 μl aliquots of the filtrates, respectively, are applied to the chromatographic plates in the form of band by Linomat 5.

3.3.4. Chromatography

TLC was performed on the 20 x 10 cm plates cut from the 20 cm x 20 cm aluminium backed plates, precoated with silica gel $60F_{254}$ (Merck, Darmstadi, Germany). Separation of the examined compounds was performed using methanol-toluene- dichloroethane-ammonia 2:3:3:0.1 (v/v/v/v) as mobile phase. Ascending development mode was performed in the twin-trough chromatographic chamber, which was pre-saturated with mobile phase vapors for 15 min. The developed chromatographic plates were dried in air and densitometrically scanned at the wavelengths 260 and 280 nm.

4. Results and Discussion

4.1. Estimation of lipophilicity and retention behavior of alpha adrenergic and imidazoline receptors ligands

4.1.1. Determination of lipophilicity indices by RP-TLC

The retention behavior of compounds in individual chromatographic systems depends on the nature of various different interactions between the solute and mobile phase, and solute and stationary phase. In TLC, the influence of the mobile phase composition and the type of stationary phase on estimation of lipophilicity has been widely investigated. The literature data reveal that octadecyl-modified silica (RP-18) is the most frequently used stationary phase, [24-27] while the methanol-water binary system presents the most common mobile phase, which has been successfully used for the estimation of lipophilicity of numerous compounds. [24, 25] Concerning the mode of chromatographic development in estimation of lipophilicity, either vertical or horizontal were utilized. [26, 31, 34, 102, 126]

For estimation of lipophilicity of our alpha adrenergic and imidazoline receptors ligands two stationary phases were selected: RP-18 and CN-modified silica while methanol and THF were used as organic modifier. Because of satisfactory obtained mobility of our investigated compounds in the methanol-water binary system and RP-18 stationary phase, different volume fractions of methanol in water were used in order to derive the retention parameter R_M^0. Our investigated compounds have basic characteristics and by using selected software program [127] calculated pK_a values are found higher than 5.35. Therefore, the influence of the solvent pH value on the retention behavior was also examined. Adding 5 vol % ammonia to the methanol-water mobile phase significantly changed an appearance of the spots. Reduced ionization of the compounds resulted in compact zones, without any tailing. However, because of a strong retention of imidazoline derivatives (naphazoline, oxymetazoline, tramazoline, and xylometazoline) in the methanol-ammonia-water system, methanol was replaced by tetrahydrofuran which is a stronger eluent on C_{18} silica. In studies of Šegan et al. [27] better reproducibility and linear dependence was also obtained by using tetrahydrofuran-ammonia-water mixture for both the C18 silica and CN stationary phase.

In our tested chromatographic systems (i.e., methanol-water/RP-18, tetrahydrofuran-ammonia-water/RP-18, and tetrahydrofuran-ammonia-water/CN) different volume fraction of organic components were examined in the ranges which enable reliably

calculation of R_f values. The lowest volume fractions of organic solvents which have been used were φ: 0.55 for methanol-water/RP-18 and tetrahydrofuran-ammonia-water/RP-18 systems, and φ: 0.60 for the tetrahydrofuran-ammonia-water/CN system. In all investigated systems the retention of compounds decreased with an increasing percent of an organic modifier in mobile phase.

For each solute, linear relationships with high correlation coefficients were established between the R_M values and the volume fraction of the organic mobile phase modifier. The obtained results for slope (m) and intercept (R_M^0) values, correlation coefficients (r), and standard errors (SE) for each investigated system (i.e., methanol-water/RP-18, tetrahydrofuran-ammonia-water/RP-18, and tetrahydrofuran-ammonia-water/CN) are shown in Tables 4, 5, and 6.

Table 4. Intercepts (R_M^0), slopes (m), correlation coefficients (r), and standard error (SE) of the TLC equation, $R_M = R_M^0 + m\varphi$ obtained for the methanol-water/RP-18 system

Compound	**R_M^0**	**m**	**r**	**SE**
Moxonidine	2.409	-3.116	0.988	0.044
Brimonidine	1.746	-1.746	0.987	0.026
Tizanidine	2.558	-3.064	0.996	0.026
Clonidine	1.439	-1.657	0.985	0.026
Amiloride	2.168	-2.063	0.972	0.046
Guanfacine	1.669	-1.421	0.978	0.027
Guanabenz	2.883	-2.965	0.982	0.052
Idazoxan	2.444	-2.858	0.990	0.037
Efaroxan	2.738	-3.344	0.993	0.035
Harmine	2.852	-2.706	0.993	0.030
Harmane	2.935	-2.921	0.981	0.053
Naphazoline	2.627	-2.725	0.985	0.043
Tetrahydrozoline	2.237	-2.668	0.991	0.033
Tramazoline	2.280	-2.689	0.987	0.040
Oxymetazoline	2.401	-2.565	0.995	0.024
Xylometazoline	2.865	-3.159	0.991	0.038

Table 5. Intercepts (R_M^0), slopes (m), correlation coefficients (r), and standard error (SE) of the TLC equation, $R_M = R_M^0 + m\varphi$ obtained for tetrahydrofuran-ammonia-water/RP-18 system

Compound	**R_M^0**	**m**	**r**	**SE**
Moxonidine	0.715	-1.971	0.971	0.044
Brimonidine	0.605	-1.788	1.000	0.004
Tizanidine	2.000	-3.282	0.980	0.061
Clonidine	2.323	-3.657	0.999	0.012
Amiloride	0.907	-2.080	0.995	0.019
Guanfacine	2.062	-3.458	0.998	0.022
Guanabenz	2.422	-3.851	0.999	0.017
Idazoxan	2.310	-3.598	0.990	0.046
Efaroxan	2.724	-4.034	0.998	0.025
Harmine	2.410	-3.813	0.999	0.016
Harmane	2.368	-3.702	0.999	0.013
Naphazoline	2.568	-3.325	0.994	0.032
Tetrahydrozoline	2.486	-3.117	0.992	0.035
Tramazoline	2.487	-2.916	0.991	0.035
Oxymetazoline	2.879	-3.665	0.993	0.039
Xylometazoline	3.197	-3.900	0.990	0.050

Table 6. Intercepts (R_M^0), slopes (m), correlation coefficients (r), and standard error (SE) of the TLC equation, $R_M = R_M^0 + m\varphi$ obtained for the tetrahydrofuran-ammonia-water/CN system

Compound	R_M^0	m	r	SE
Moxonidine	0.274	-1.456	0.990	0.019
Brimonidine	0.386	-1.551	0.973	0.034
Tizanidine	1.329	-2.580	0.994	0.026
Clonidine	1.691	-3.061	0.991	0.038
Amiloride	0.884	-1.764	0.995	0.015
Guanfacine	1.327	-2.709	0.992	0.032
Guanabenz	1.758	-3.257	0.990	0.043
Idazoxan	1.516	-2.819	0.987	0.042
Efaroxan	1.860	-3.173	0.994	0.033
Harmine	1.651	-3.075	0.987	0.045
Harmane	1.980	-3.602	0.979	0.069
Naphazoline	1.989	-2.643	0.994	0.027
Tetrahydrozoline	2.015	-2.465	0.983	0.042
Tramazoline	2.124	2.550	0.978	0.050
Oxymetazoline	2.551	-3.298	0.993	0.034
Xylometazoline	2.580	-3.176	0.993	0.033

The results given in Table 4, 5, and 6 show that R_M^0 values obtained in three examined systems are different. These differences could be result of various interactions appearing between organic modifier from mobile phase and unmodified silanol groups presented on the surface of RP-18 and CN stationary phases. Also, in addition to the interactions between tested compounds and functional groups on stationary phases, retention behavior depends also on specific interactions between examined compounds and component of mobile phase. In this study majority of compounds have the highest R_M^0 values in methanol which can be attributed to its lower elution strength compared to tetrahydrofuran. Moreover, all compounds exhibit lower R_M^0 values in tetrahydrofuran-ammonia water/CN than in tetrahydrofuran-ammonia-water/RP-18 system. Such

chromatographic behaviour can be a consequence of stronger hydrophobic interactions between the compounds and the alkyl chains of octadecyl silica, than with the less hydrophobic cyano propyl groups of the CN stationary phase.

Agonists of α_1-adrenergic receptors (such, as 2-imidazolines naphazoline, oxymetazoline, xylometazoline, tramazoline and tetrahydrozoline) demonstrate higher affinity to and stronger retention on both, the C_{18} and CN stationary phases, when compared with the α_2-adrenergic receptor agonist and the IR-ligands, clonidine, moxonidine, tizanidine and brimonidine (which belong to the 2-aminoimidazolines derivatives). Among the guanidines derivatives, guanabenz lacking carbonyl group attached to the nitrogen atom of guanidine exhibits stronger retention, when compared with amiloride and guanfacine. Similar chromatographic behavior was observed between harmane and harmine, which differ with the methoxy group only, present at position 7 of β-carboline, harmine. Also a higher retention of efaroxan than that of idazoxan can be ascribed to the presence of the bicyclic 2-ethyl-2.3-dihydrobenzofuran moiety in the efaroxan structure.

In addition to R_M^0 values another chromatographic parameters such as slope **(m)** and **C_0,** can be also used for estimation of compounds lipophilicity. In order to examine the possibility of their application in case of our examined alpha adrenergic and imidazoline receptors ligands, relationships between the slope **(m)** and the intercept (R_M^0) has been studied for all examined systems. The results of these calculations were present in Table 7.

Table 7. Relationships obtained between intercept (R_M^0) and slope (m)

Chromatographic system	**Equation**	**r**	**r^2**	**SE**
CH_3OH-H_2O/RP-18	m = -1.110 R_M^0 - 0.050	0.877	0.769	0.287
THF-NH_3-H_2O/RP-18	m = -0.854 R_M^0 - 1.420	0.904	0.817	0.317
THF-NH_3-H_2O/CN	m = -0.784 R_M^0 - 1.429	0.819	0.670	0.378

The best correlation was obtained for THF-NH_3-H_2O/RP-18 chromatographic system indicated on possibility of using slope for estimation of lipophilicity while the lowest was noticed in CH_3OH-H_2O/RP-18 system where this parameter is less reliable for estimation of compounds

lipophilicity. Like in the work of Šegan et al. [27] high correlation between these parameters confirm uniformity in the retention process.

In addition, correlation between R_M^0 and C_0 was also examined (obtained values for C_0 are presented in Table S1). Equations describing these relationships along with obtained statistical parameters are shown in Table 8.

Table 8. Relationships obtained between intercept (R_M^0) and C_0 values

Chromatographic system	**Equation**	**r**	**r^2**	**SE**
CH_3OH-H_2O/RP-18	$C_0 = -0.0361R_M^0 + 1.017$	0.152	0.023	0.110
THF-NH_3-H_2O/RP-18	$C_0 = 0.187R_M^0 + 0.236$	0.923	0.852	0.061
THF-NH_3-H_2O/CN	$C_0 = -0.784R_M^0 - 1.429$	0.916	0.839	0.078

Statistical parameters presented in Table 8 show that there is no relationship between R_M^0 and C_0 values in the CH_3OH-H_2O/RP-18 chromatographic system. On the other side, high correlations were achieved in the THF-NH_3-H_2O/RP-18 and THF-NH_3-H_2O/CN chromatographic systems indicated that C_0 values obtained using tetrahydrofuran-ammonia-water as mobile phase could be used for evaluation of lipophilicity of imidazoline and alpha adrenergic receptors ligands.

Vertical mode of development was applied in the above mentioned chromatographic systems. The retention behavior of imidazoline and alpha adrenergic receptors ligands was also examined by horizontal mode of development. For this study investigation was started with tetrahydrofuran – ammonia –water / RP – 18 chromatographic system using the same volume fraction of organic modifier as in vertical mode (φ: 0.6-0.8). This system was initially chosen because statistical parameters obtained by applying vertical mode of development showed the best correlation between chromatographic indices (r: 0.904 between *m* and R_M^0, and r: 0.923 between R_M^0 and C_0).

The obtained slope (m), intercept (R_M^0), C_0 values, correlation coefficients (r), and standard errors (SE) for tetrahydrofuran – ammonia –water / RP – 18 chromatographic system using horizontal mode of development are listed in Table 9.

Table 9. Intercepts (R_M^0), slopes (m), C_0, correlation coefficients (r), and standard error (SE) of the TLC equation, $R_M = R_M^0 + m\varphi$ obtained by horizontal development

Compound	Tetrahydrofuran-ammonia-water/RP-18				
	R_M^0	m	C_0	r	SE
Moxonidine	1.073	-2.691	0.399	0.986	0.042
Brimonidine	0.622	-1.800	0.346	0.997	0.012
Tizanidine	3.399	-5.728	0.593	0.961	0.149
Clonidine	2.342	-3.976	0.589	0.993	0.044
Amiloride	1.525	-2.980	0.512	0.985	0.047
Guanfacine	2.507	-4.386	0.572	0.995	0.041
Guanabenz	2.889	-4.841	0.597	0.974	0.102
Idazoxan	2.100	-3.557	0.590	0.984	0.067
Efaroxan	1.735	-2.958	0.587	0.989	0.040
Harmine	2.801	-4.665	0.600	0.983	0.079
Harmane	2.676	-4.445	0.602	0.991	0.054
Naphazoline	1.974	-2.388	0.827	0.990	0.030
Tetrahydrozoline	1.887	-2.177	0.867	0.985	0.035
Tramazoline	2.420	-2.660	0.910	0.965	0.066
Oxymetazoline	2.797	-3.445	0.812	0.974	0.073
Xylometazoline	3.071	-3.553	0.864	0.976	0.071

Correlations between obtained chromatographic indices R_M^0 and m, as well as R_M^0 and C_0 have been also examined and are presented in Table 10.

Table 10. Relationships obtained between R_M^0 and m; and R_M^0 and C_0

Equitations	**r**	**r^2**	**SE**
$m = -1.170R_M^0 - 0.897$	0.800	0.640	0.677
$C_0 = 0.113R_M^0 + 0.389$	0.502	0.252	0.150

By comparing the statistical results obtained by applying different mode of development (horizontal and vertical) it can be seen that no improvements were achieved using horizontal development regarding correlation coefficients between R_M and φ; R_M^0 and *m*; and R_M^0 and C_0 (Tables 4, 5 , 6 , 7, 8, 9, 10). Therefore, further examination using methanol-water/RP-18 and tetrahydrofuran-ammonia-water/CN systems was not performed.

4.1.2. Correlation between the chromatographic indices and calculated log *P* values

Chromatographic parameters have been widely used as an alternative to log *P*, because chromatographic system can be viewed in terms of partitioning of an analyte between a polar aqueous mobile phase and a nonpolar stationary phase. Numerous studies have shown significant correlation between retention parameters (R_M^0, m and C_0) of series of related compounds obtained by RP-TLC and log *P* values calculated by different softwares. [26, 31, 34, 35, 102]

Because of the importance of hydrophobicity and log *P* for QSAR and drug discovery, a larger number of different calculation methods have been derived for estimating octanol-water partition coefficient (log *P*) of compounds. Recently Mannhold has divided all these methods into three groups: fragmental, atom-based, and conformation dependent.

In fragmental-based methods, the log *P* values are calculated by cut the molecules into different fragments with application of correction factors to estimate and incorporate molecular environment effects. The total log *P* for the molecule can be calculated as a sum of all these contributions. The best accuracy was produced by fragmental-based methods.[127]The most common examples of the fragment-based method are ACD/log *P*, KOWWIN log *P*, Milog *P*, Clog *P*. [128]

On the other hand in the atom-based method, the calculation of log *P* (Xlog *P2*, Xlog*P3*, Alog *P*, AClog*P*) are achieved by cutting down molecules to the single atoms and often without applying corrections. The difference between two methods led to differentiate between absolute log *P* values. The main advantages of the substructure methods (fragment and atom-based), are their highly interpretable results and the ability to train the calculation algorithm with few experimental data.

Conformation dependent method used the description of the entire molecule and include: empirical, methods based on molecule's 3D-structure or methods based on topological descriptors (Alog *P*s, Mlog *P*) to quantify log *P*. [129]

Some of log *P* values calculated for the 16 examined compounds and obtained by different methods are given in Table 11.

Table 11. The calculated log *P* values for the investigated compounds

Compound	Alog P_s	AClog *P*	milog *P*	Alog *P*	Mlog *P*	KOWWIN log *P*	Xlog *P2*	Xlog *P3*	Clog *P*	ACD/log *P*	ChemAxon log *P*
Moxonidine	0.75	0.73	-0.05	0.99	1.63	0.27	0.70	0.93	1.31	0.33	0.93
Brimonidine	1.40	1.30	1.17	1.63	1.05	0.61	1.55	0.96	1.51	1.20	0.77
Tizanidine	1.69	1.98	1.33	2.21	1.23	0.60	2.13	1.48	2.14	2.39	1.40
Clonidine	1.92	2.39	1.92	2.74	2.66	1.89	2.57	1.55	1.43	2.36	1.84
Amiloride	-0.48	-1.66	-1.24	-0.78	0.26	0.09	0.17	0.06	0.11	0.93	-0.43
Guanfacine	2.28	1.21	1.56	2.00	2.30	1.31	2.20	2.24	1.37	1.33	1.57
Guanabenz	1.69	1.71	2.40	2.36	2.68	1.70	3.73	1.95	2.98	2.66	2.13
Idazoxan	1.01	0.77	1.03	1.19	1.18	1.77	1.18	0.70	1.81	2.31	1.06
Efaroxan	2.98	1.77	2.49	2.19	2.27	3.18	1.79	1.71	2.84	3.10	2.14
Harmine	3.05	2.67	2.63	2.44	1.29	2.83	2.51	3.56	3.13	3.05	1.85
Harmane	3.36	2.78	2.59	2.45	1.59	2.75	2.59	3.59	3.06	3.06	2.10
Naphazoline	3.44	2.25	2.85	2.26	2.93	3.52	2.76	2.07	3.83	2.99	2.63
Tetrahydrozoline	3.11	2.06	2.26	2.38	2.84	3.69	2.27	1.79	3.54	2.85	2.55
Tramazoline	1.88	2.29	1.82	2.93	2.68	2.56	2.57	2.36	2.49	2.78	2.03
Oxymetazoline	3.70	2.92	3.86	3.45	3.04	4.87	3.36	2.86	4.61	3.76	3.91
Xylometazoline	4.68	3.21	4.15	3.72	3.61	5.35	4.19	3.22	5.38	4.59	4.19

Ideally, the correlation between the calculated log *P* values, although they are differ from each other, should be complete ($r = 1$) regardless of the applied algorithm. However, results in Table S2 show that correlation coefficients obtained between the log *P* values calculated using different software are in the range from 0.512 (between Mlog *P* and Xlog *P3* values) to 0.961 (between AClog *P* and Alog *P* values) which is as previously explained results of different procedures applied for calculation of log *P* values.

In order to examine usefulness of the retention parameters (R_M^0, m and C_0) for an assesment of lipophilicity of alpha adrenergic and imidazoline receptor ligands, the experimentally determined retention constants were correlated with the log *P* values calculated with use of several softwares. [114-118]

In the case of parameter R_M^0, satisfactory correlations between the R_M^0 values experimentally measured in the tetrahydrofuran-ammonia-water/RP-18 and the tetrahydrofuran-ammonia-water/CN systems on the one hand, and the calculated log *P* values for the set of the 16 investigated compounds on the other were obtained. Statistical parameters of these relationships are given in Table 12.

Table 12. Parameters *a* and *b*, correlation coefficient *r* values, and standard error (SE) values for the linear relationship $R_M^0 = a + b \log P_{calc}$ calculated for the (A) THF-NH_3-H_2O/RP-18 and (B) THF-NH_3-H_2O/CN chromatographic systems

	A		B					
	a	*b*	*r*	*SE*	*a*	*b*	*r*	*SE*
Alog P_s	1.107	0.459	0.788	0.483	0.710	0.399	0.783	0.428
AClog *P*	1.319	0.471	0.728	0.538	0.919	0.395	0.698	0.492
milog *P*	1.234	0.479	0.847	0.418	0.827	0.413	0.833	0.380
Alog *P*	0.958	0.560	0.776	0.496	0.597	0.479	0.758	0.448
Mlog *P*	0.902	0.603	0.731	0.536	0.515	0.532	0.737	0.464
KOWWIN log *P*	1.211	0.408	0.847	0.417	0.749	0.377	0.894	0.308
Xlog *P2*	0.878	0.563	0.777	0.494	0.496	0.496	0.782	0.428
Xlog *P3*	1.174	0.505	0.680	0.576	0.753	0.447	0.687	0.499
Clog *P*	1.015	0.439	0.786	0.486	0.586	0.399	0.815	0.398
ACD/log *P*	0.570	0.639	**0.916**	0.315	0.208	0.569	**0.933**	0.247
ChemAxon Log *P*	1.086	0.557	0.830	0.438	0.661	0.500	0.851	0.361

The most significant correlations for the tetrahydrofuran-ammonia-water system, and the RP-18 and CN stationary phases were obtained with the log *P* values calculated by the ACD/log *P* program (r equal to 0.916 and 0.933, respectively, for RP-18 and CN stationary phases). The linear relationship obtained between the R_M^0 values experimentally measured in the tetrahydrofuran-ammonia-water/RP-18 and the tetrahydrofuran-ammonia-water/CN systems, and the calculated log *P* values by the ACD/log *P* program are given in Figure 11.

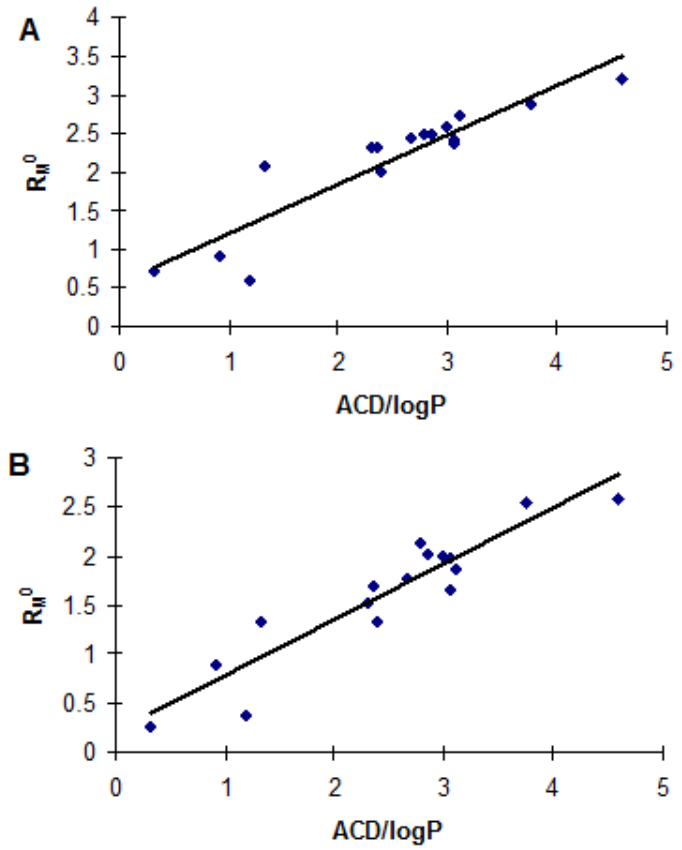


Figure 11. Correlation between R_M^0 and ACD/log*P* values obtained for (A)THF-NH_3-H_2O/RP-18, (B) THF-NH_3-H_2O/CN chromatographic system.

Good correlations (r > 0.83) were also observed with milog *P*, KOWWIN log *P*, and ChemAxon log *P*, while the worst dependence was obtained with Xlog *P3* (r equal to 0.680 and 0.687, respectively, for RP-18 and CN stationary phases). It can be conclude that the best agreement was obtained by use of fragmental- based method (KOWWIN log *P*, Milog *P*, ACD log *P),* while the worst correlation was obtained with the atom- based method (Xlog *P3).* Moreover, from the results given in Table 12, it can be seen that the statistical characteristics of the linear relationship R_M^0 = f (log *P*) obtained for the two chromatographic systems, THF-NH_3-H_2O/RP-18 and THF-NH_3-H_2O/CN, are similar. This similarity is fully confirmed, when the R_M^0 values originating from these two chromatographic systems are plotted against each other (i.e., $R_M^0{}_{CN}$ = f ($R_M^0{}_{RP\text{-}18}$), as given below.

$$R_M^0{}_{CN} = -0.183 + 0.837\ R_M^0{}_{RP\text{-}18}\ (r = 0.957) \qquad (17)$$

A comparison of the retention constants R_M^0 obtained in the methanol-water/RP-18 system with the calculated log *P* values from Table 11 indicated that the correlation coefficients are not always too high ($r < 0.6$), Table S3. The highest correlation was obtained with Clog *P* values ($r = 0.580$) and their linear dependence can be represented by the following equation:

$$R_M^0 = 1.885 + 0.195 \text{ Clog } P \quad (r = 0.580, SE = 0.384) \qquad (18)$$

This discrepancies between the magnitude of the correlation coefficients obtained for the different mobile/stationary phase pairs is probably a consequence of the different types of the solute-stationary phase and the solute-mobile phase interactions affecting the retention in a given chromatographic system. Besides, a pronounced ionization of all investigated compounds in the methanol-water system can additionally contribute to a lower reliability of the estimated lipophilicity, keeping in mind that the partition coefficient relates to the neutral form of a compound.

Besides R_M^0 value, usefulness of the other chromatographic parameters (*m* and C_0) for estimation of compounds lipophilicity was also examined. Correlation coefficients obtained by comparing *m* and C_0 values obtained in THF-NH_3-H_2O/RP-18 and THF-NH_3-H_2O/CN chromatographic systems with calculated log *P* values are presented in Table 13.

Table 13. Correlations between retention parameters (*m* and C_0) and calculated log *P* values for (**A**) THF-NH_3-H_2O/RP-18 and (**B**) THF-NH_3-H_2O/CN chromatographic systems

Calculated log *P*	**A**		**B**	
	m	C_0	m	C_0
Alog P_s	-0.661	0.710	-0.673	0.627
AClog *P*	-0.634	0.659	-0.674	0.505
milog *P*	-0.740	0.743	-0.763	0.641
Alog *P*	-0.646	0.727	-0.673	0.590
Mlog *P*	-0.521	0.754	-0.494	0.681
KOWWIN log *P*	-0.635	0.829	-0.650	**0.804**
Xlog *P2*	-0.681	0.697	-0.722	0.613
Xlog *P3*	-0.636	0.588	-0.723	0.480
Clog *P*	-0.597	0.749	-0.611	0.695
ACD/log *P*	**-0.776**	**0.847**	**-0.799**	0.801
ChemAxon log *P*	-0.647	0.787	-0.660	0.710

The obtained results (Table 13) revealed that parameters *m* and C_0 determined for THF-NH_3-H_2O/RP-18 system have the best correlation with ACD/log *P* value (r: -0.776 and r: 0.847 for parameters *m* and C_0, respectively) which is exactly same case as for the R_M^0 value. Something different situation was observed for the THF-NH_3-H_2O/CN system. In this system, parameter *m*, same as R_M^0, showed the best correlation with ACD/log *P* value (r: -0.799) while parameter C_0 the best correlates with KOWWIN log *P* value (r: 0.804). Despite satisfactory correlations obtained for *m* and C_0 values, it can be concluded that for both chromatographic systems the most reliable parameter for assessment of lipophilicity of I-IR/α-AR ligands is R_M^0 value.

In the case of CH_3OH-H_2O/RP-18 system linear relationships were not noticed, therefore parameters *m* and C_0 cannot be used for estimation of lipophilicity of I-IR/α-AR ligands using this chromatographic system.

4.1.3. Principal component analysis

In order to examine similarity and differences in lipophilicity between the tested compounds as well as between experimentally determined and calculated lipophilicity parameters, PCA was performed in Simca P + 12.0 program [124] For this chemometric analysis R_M^0 values obtained for the THF-NH_3-H_2O/RP-18 and THF-NH_3-H_2O/CN chromatographic system, which proved to be the most reliable systems for assessing compounds lipophilicity, along with calculated log *P* values were used as input data. The analyzed data were organized in matrix X (16 x 13) where rows represent studied compounds (16) while columns represent examined variables (R_M^0 values and calculated log *P* values).

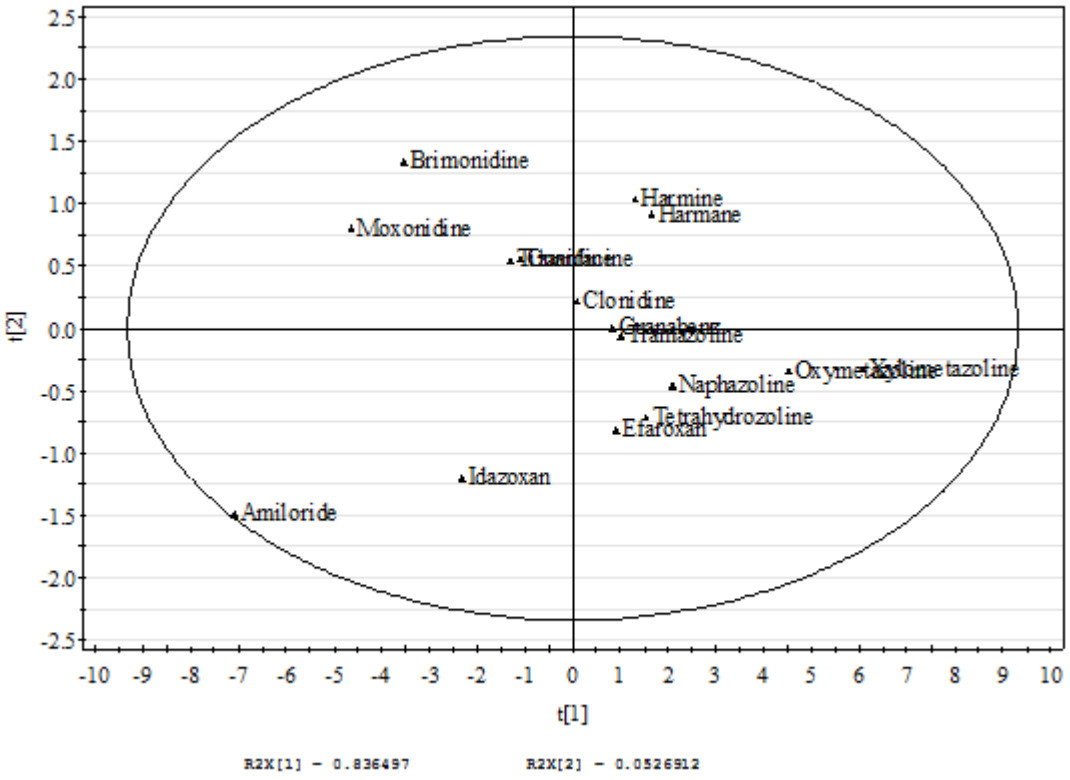


Figure 12. Score plot of PC1 and PC2 as a result of PCA for the retention parameter R_M^0 and calculated log *P* values**.**

According to the first principal component, PC1 which describe 83.65% of the data variance, R_M^0 values and calculated log *P* values are grouped in the same cluster (p[1] >0.24 (Figure 12). These parameters can be distinguished based on PC2 which describes only 5.27% of data variance. Approximately half of the calculated log *P* values, **group I** (Xlog *P3*, AClog *P*, Alog *P*, Xlog *P2*,

Alog *P*s, and milg *P*) are formed the cluster with p[2] >0 while the rest of calculated log *P* values, **group II** (ChemAxon log *P*, Clog *P*, Mlog *P*, ACD/log *P*, KOWWIN log *P*) are in the same cluster with experimentally determined chromatographic parameters R_M^0 and with p[2] <0 (Figure 13.).

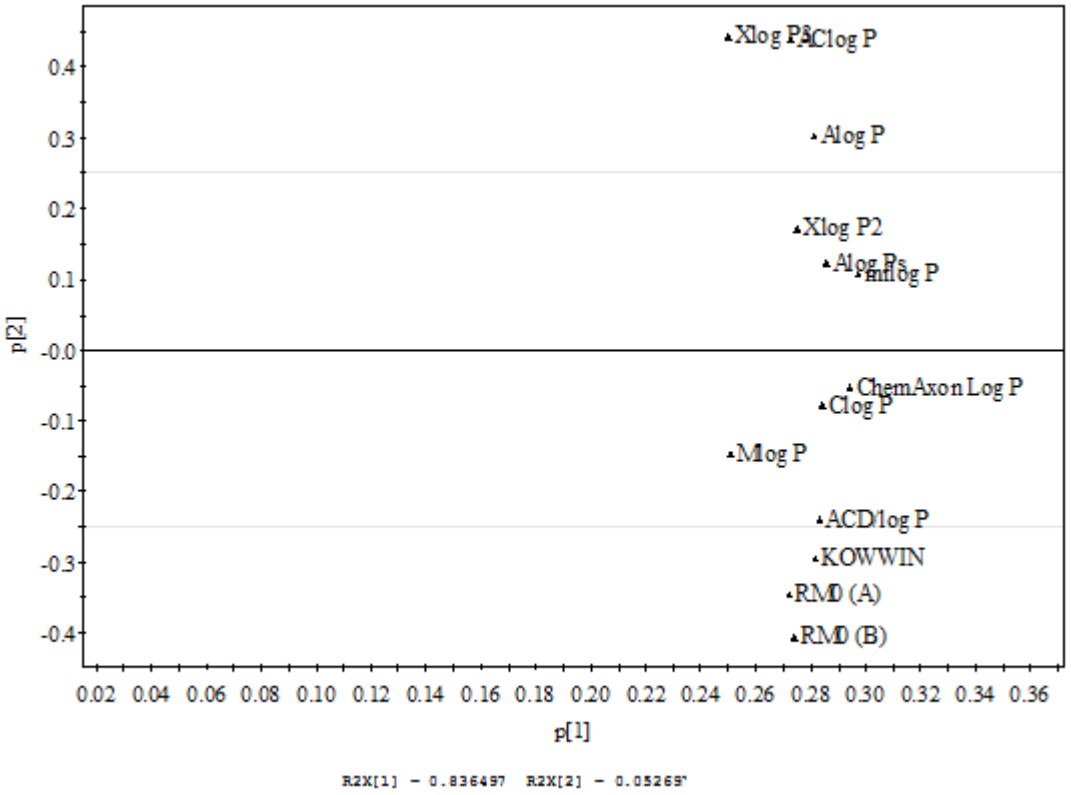


Figure 13. Loading plot as a result of PCA for the retention parameter R_M^0 and calculated log *P* values.

According to the score plot (Figure 12.) 16 examined compounds are classified into different lipophilicity groups. Compounds on the right side of the plot (harmane, harmine, clonidine, guanabenz, tramazoline, naphazoline, xylometazoline, oxymetazoline, tetrahidrozoline, and efaroxan) possess high values of calculated and experimental lipophilicity indices.

Harmane, harmine, clonidine and guanabenz are in the same group based on the something higher values of group I of calculated log *P* values, while tramazoline, naphazoline, xylometazoline, oxymetazoline, tetrahidrozoline, and efaroxan are grouped together based on the something higher R_M^0 values and group II of calculated log *P* values. Compounds on the left side of the plot (brimonidine, moxonidine, tizanidine and guanfacine, amiloride and idazoxan) are grouped on the basis of the lowest values of all parameters.

4.1.4. QSRR study

The QSRR modeling of 16 alpha adrenergic and imidazoline receptor ligands was performed with aid of the PLS regression. In the QSRR study, experimentally obtained retention parameters (R_M^0) in the three different chromatographic systems were used as dependent variables, while the computed molecular parameters of the examined compounds were used as independent variables. The earlier described training set of 12 compounds and the test set of 4 compounds were used for building of the QSRR models. Structures of the test set were randomly selected, but taking into the account that each chemical group (i.e., the 2-aminoimidazoline, guanidine, 2-arylmethylimidazoline and β-carboline derivatives) has one representative in the test set.

The statistical results of the created QSRR models are given in Table 14.

Table 14. Statistical results of the developed QSRR models

Chromatographic system	Selected descriptors	R^2Y	Q^2	RMSEE	RMSEP
CH_3OH-H_2O/RP-18	Clog *P*, AMW, P_VSA_v_3	0.710	0.640	0.247	0.694
THF-NH_3-H_2O/RP-18	ACD/log *P*, nN, P_VSA_e_2	0.958	0.923	0.171	0.339
THF-NH_3-H_2O/CN	ACD/logP, nON, P_VSA_e_2	0.934	0.873	0.186	0.218

The best statistical parameters were obtained for THF-NH_3-H_2O/RP-18 chromatographic system, while the lowest power for prediction of the test set showed QSRR (CH_3OH-H_2O/RP-18) model (RMSEP: 0.694). However, in all models criterion for high values of Q^2 (Q^2 >0.5) and low errors for the training and the test set (RMSEE and RMSEP) were satisfied indicated on their good prognostic capacity [125] (Table 14).

Graphs of the observed versus predicted R_M^0 values resulting from the selected models also showed high correlation between experimentally obtained R_M^0 and those predicted by created models (Figure 14.).

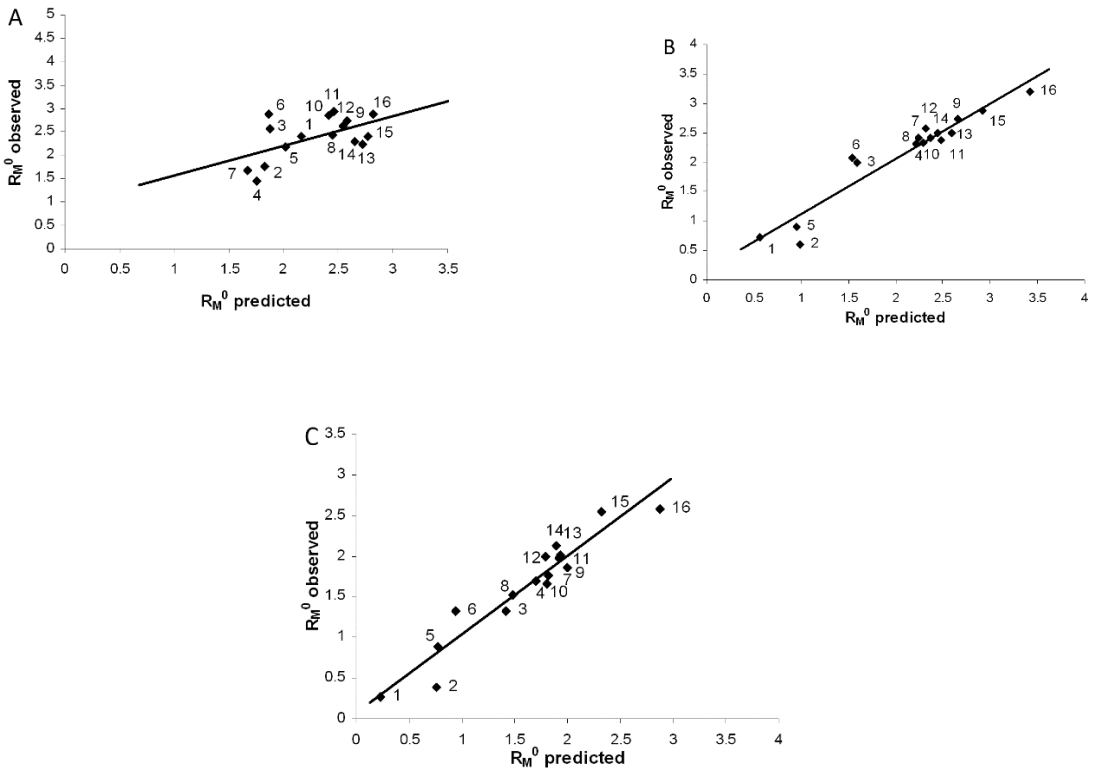


Figure 14. Correlations between the observed and the predicted R_M^0 values for the selected QSRR models in the (A) CH_3OH-H_2O/RP-18, (B) THF-NH_3-H_2O/RP-18 and (C) THF-NH_3-H_2O/CN chromatographic systems.

In addition, performed Y-scrambling test was shown that models were not obtained by chance correlation (Figure 15.). The following intercept values of the R^2 and Q^2 were obtained (R^2: 0.038 and Q^2: -0.25; R^2: 0.0725 and Q^2: -0.391; R^2: 0.0351 and Q^2: -0.373, for CH_3OH-H_2O/RP-18, THF-NH_3-H_2O/RP-18 and THF-NH_3-H_2O/CN chromatographic systems, respectively) which were in accordance with criterion lower than 0.3–0.4 for R^2-intercept and 0.05 for Q^2-intercept. [125]

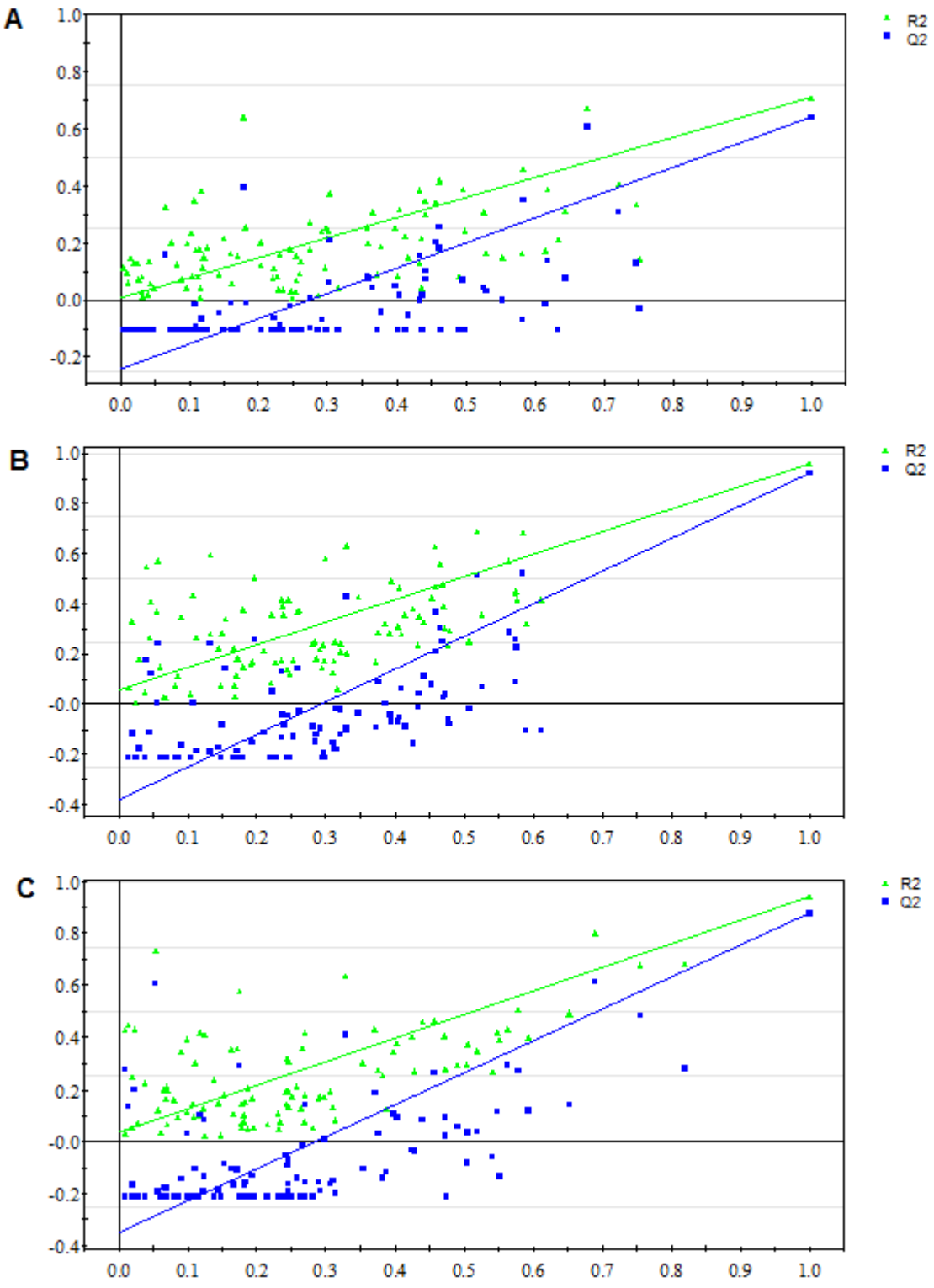


Figure 15. Y-scrambling test for: (A) CH_3OH-H_2O/RP-18, (B) THF-NH_3-H_2O/RP-18 and (C) THF-NH_3-H_2O/CN chromatographic systems.

As can been seen in Table 14 optimal QSRR models for all examined systems were created with three descriptors. They have been selected from the large number of calculated descriptors according to their VIP values. Only the variables with the VIP values higher than 0.5 have been considered for the regression while those with lower VIP values were removed from the model until the models with the best statistical results have been obtained. The selected descriptors and their VIP values are presented in Figure 16.

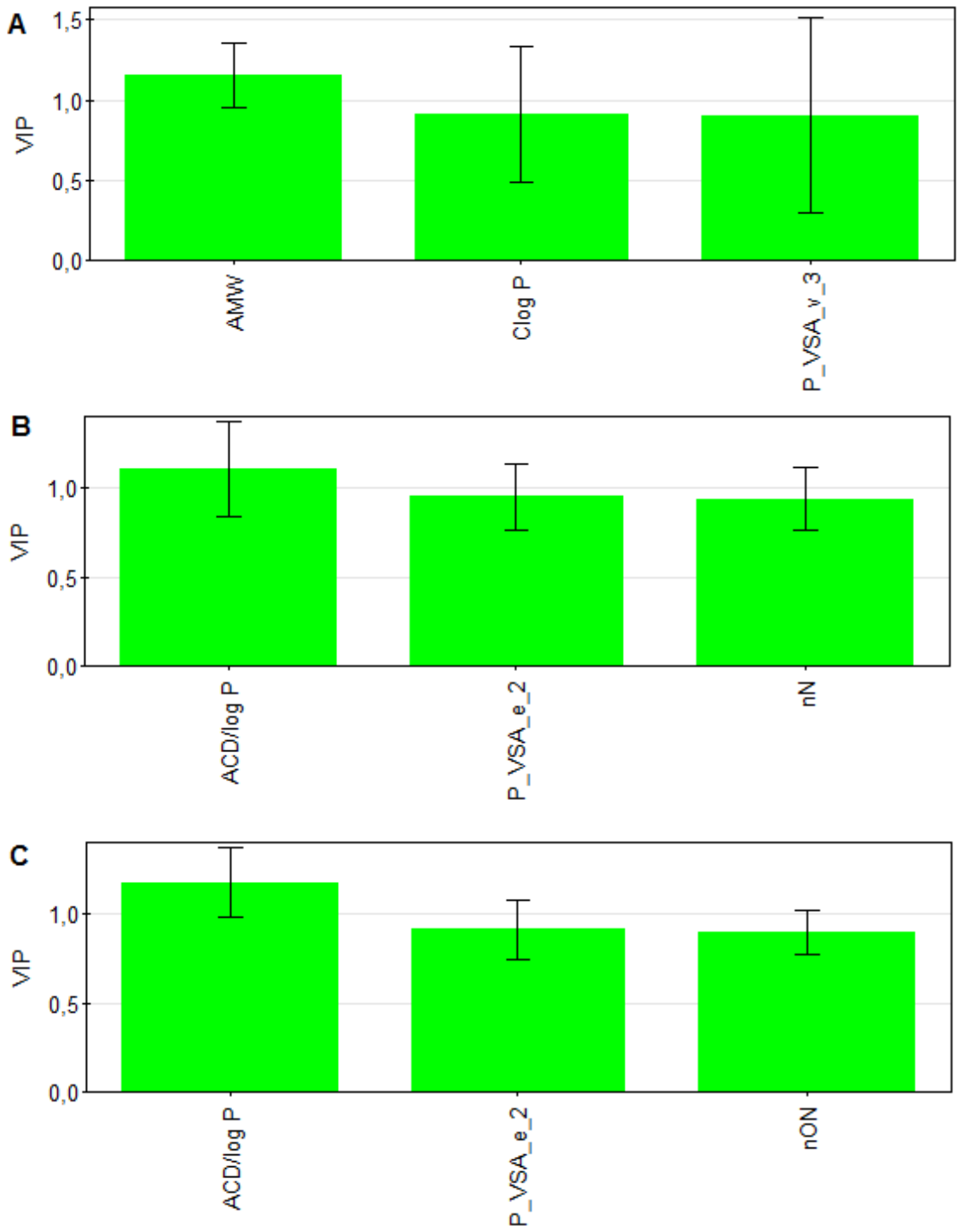


Figure 16. VIP values of most important descriptors used in QSRR study: (A) CH_3OH-H_2O/RP-18, (B) THF-NH_3-H_2O/RP-18 and (C) THF-NH_3-H_2O/CN chromatographic systems.

Besides importance of molecular descriptors expressed through the VIP values, illustrative representation of impact of selected descriptors on dependent variables can be seen on the plot of coefficients (Figure 17.). Descriptors with positive influence on dependent variable are on the upper side of x-axis, while descriptors with negative influence on dependent variable are on the bottom side of the x-axis.

The values of selected descriptors are listed in Table S4.

In all devised QSRR models, logarithm of the partition coefficient, log *P* (Clog *P* and ACD/log *P*) is selected as the important property with positive influence on the retention in the tested chromatographic RP-TLC systems. Beside Clog *P*, the average molecular weight (AMW) [122] and P_VSA-like on van der Waals volume, bin 3 (P_VSA_v_3) descriptors [122] were selected as molecular properties with negative correlation (Fig. 17A.) with the R_M^0 values that contribute to an overall retention behavior in the methanol-water/RP-18 chromatographic system. Similar sets of descriptors were selected for both, the RP-18 (ACD/log *P*, nN, P_VSA_e_2) and the CN (ACD/log *P*, n ON, P_VSA_e_2) stationary phases and tetrahydrofuran-ammonia-water as mobile phase. The P_VSA-like on Sanderson electronegativity, bin 2 (P_VSA_e_2) descriptor [122] is defined as an amount of the van der Waals surface area (VSA), which in certain range has the P property (Sanderson electronegativity). [123] In the tetrahydrofuran-ammonia-water/RP-18 and the tetrahydrofuran-ammonia-water/CN chromatographic systems, the P_VSA_e_2 descriptor exerts negative influence on the R_M^0 values. Therefore the compounds with higher P_VSA_e_2 values have lower retention on the two investigated stationary phases. The number of nitrogen atoms (nN) [122] is a constitutional descriptor and in the tetrahydrofuran-ammonia-water/RP-18 system, it exerts negative influence (Fig.17B.) on the retention constant (R_M^0). Thus the compounds with a higher number of the nitrogen atoms (nN) less efficiently interact with the C18-modified silica stationary phase, which results in a lower retention thereof.

The number of the hydrogen bond acceptors (nON) [115] negatively contributes (Fig.17C.) to the R_M^0 values in the tetrahydrofuran-ammonia-water/CN system, so that the larger number of the nitrogen and oxygen atoms as the hydrogen accepting sites leads to the lower retention on the CN stationary phase.

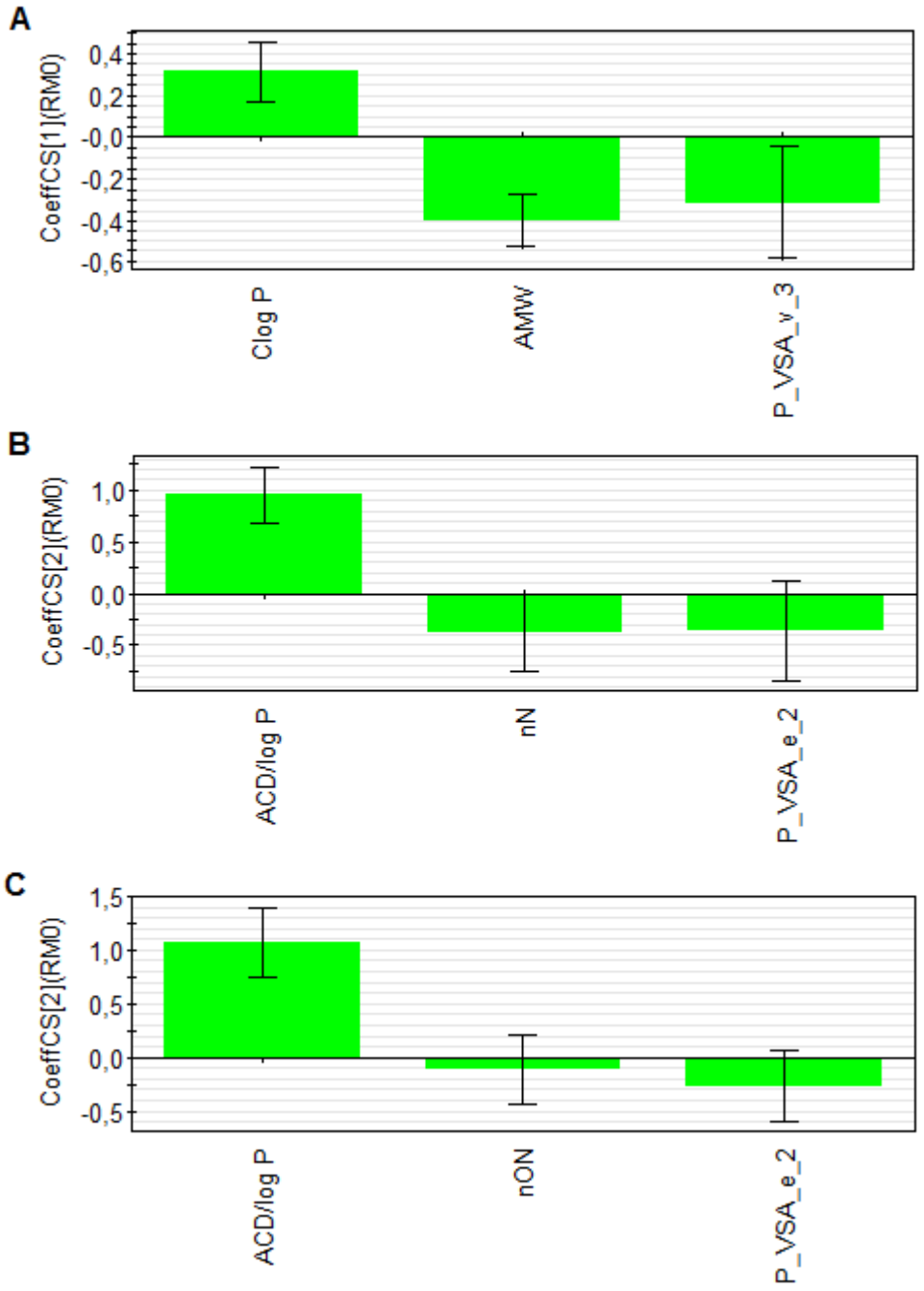


Figure 17. Plot of the coefficients of the selected descriptors for QSRR models in the (A) CH_3OH-H_2O/RP-18, (B) THF-NH_3-H_2O/RP-18 and (C) THF-NH_3-H_2O/CN chromatographic systems.

4.2. Quantitative analysis of moxonidine and its impurities

4.2.1. Optimization of chromatographic condition

In order to optimize chromatographic condition for an efficient separation of moxonidine and its four impurities, different stationary phases and different mobile phase compositions were examined. Preliminary studies have started from examination of the retention behaviour of the analytes using single non-polar (toluene) and single polar (methanol) solvent as two mono-component mobile phases, and the polar silica gel plates as stationary phase. In contrast to the retention behaviour in non-polar solvents such as toluene, where all analytes demonstrated high affinity toward stationary phase and were retained on the start line, higher mobility (especially with moxonidine) was observed with use of a polar solvent such as methanol. In order to achieve satisfactory resolution of the examined compounds, further tests were directed toward examination of the analytes retention in the toluene-methanol mixture. In order to reduce peak tailing, basic solvent such as ammonia or triethylamine (TEA) was added to mobile phase. Higher volume fraction of methanol in mobile phase resulted in a too high R_f value for moxonidine and impurity A (6-chloromoxonidine), while impurities B (4-methoxymoxonidine), C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine) were retained close to the start line. Therefore, higher proportion of toluene was selected, which significantly reduced the R_f values for moxonidine and increased resolution among the tested compounds. Different volume ratios of methanol, toluene, and TEA were tested to optimize the mobile phase composition and in the course of these experiments, considerable difference in the retention behaviour was observed between impurity 4-hydroxymoxonidine and impurity 6-chloromoxonidine. In fact, impurity 4-hydroxymoxonidine remained close to the start line, while impurity 6-chloromoxonidine migrated close to the front line. Well separated and compact zones were obtained by adding dichloroethane to the eluent mixture and the next qualitative and quantitative mobile phase composition was assumed as methanol-toluene-dichloroethane-TEA (2:3:3:0.1, v/v/v). In that case, the migration distances for moxonidine and impurities 6-chloromoxonidine, 4-methoxymoxonidine, 4-hydroxymoxonidine, and 6-desmethylmoxonidine were equal, respectively, to 41.2 mm ± 0.99%, 51.9 mm ± 0.82%, 24.2 mm ± 0.40%, 13.6 mm ± 0.87%, and 19.4 mm ± 0.74%. Later it was noticed that impurities 4-hydroxymoxonidine and 6-chloromoxonidine co-eluted with the tablet matrix, which was finally avoided by replacing TEA with an equal volume proportion of ammonia and assuming double development of the chromatogram using the same mobile phase, methanol-toluene-

dichloroethane-ammonia 2:3:3:0.1, v/v/v. Testing the elaborated mobile phase composition upon the HPTLC and HPTLC Lichrosphere Si60 plates, no better resolutions was achieved, so that the aluminium backed chromatographic plates pre-coated with silica gel 60 F_{254} were used for further optimization of TLC method. In-situ UV spectra of moxonidine and its impurities was performed with CAMAG TLC scanner II in reflectance mode. It noticed that moxonidine and impurities 6-chloromoxonidine, 4-hydroxymoxonidine, and 6-desmethylmoxonidine exhibited maximum absorbance at wavelength 280 nm, while impurity 4-methoxymoxonidine at 260 nm (Figure 18.).

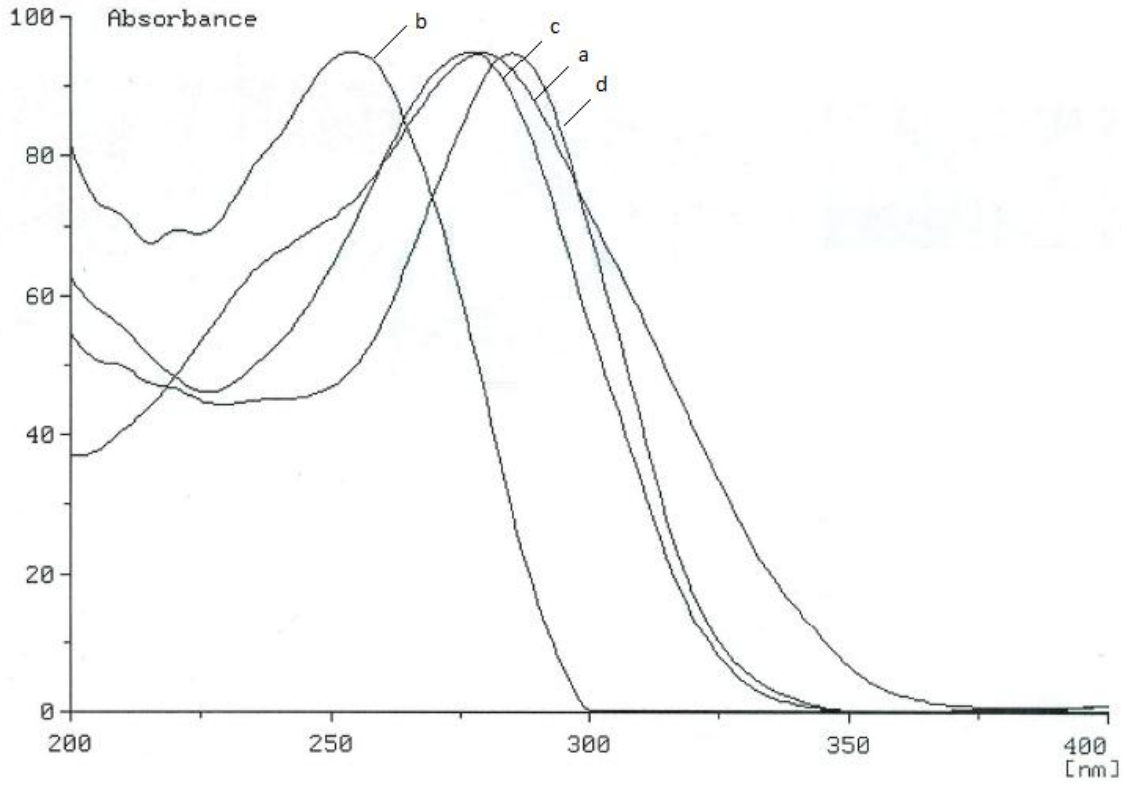


Figure 18. In situ UV spectra of impurity B (b), impurity C (c), impurity A (a), and impurity D (d), respectively**.**

4.2.1.1. Assessment of an impact of chromatographic factors on the resolution between critical peak pairs

All these experiments showed that content of the methanol in the mobile phase together with developing distance, band width and saturation time are the factors influencing resolution and the

retention behaviour of examined compounds. Therefore, these factors were selected for further, deeper investigation by experimental design. Factors and their levels are presented in Table 15.

Table 15. The examined factors and their levels

Examined factors	Measuring units	Levels		
		(-1)	(0)	(+1)
Percent of methanol in mobile phase (x_1)	%	19.7	24.7	29.7
Saturation time (x_2)	min	5	15	25
Band width (x_3)	mm	8	10	12
Developing distance (x_4)	mm	160	180	200

For the assessment of an impact of selected factors on the retention behavior of tested compounds reduced central composite face-centered design was selected with the total number of the experiments being 25, with five experiments representing replications in the central point. [130]
Resolution between impurities C (4-hydroxymoxonidine) and D (6-desmethylmoxonidine) (Rs(C/D)), and moxonidine and impurity A (6-chloromoxonidine) (Rs(M/A)) were followed as systems output obtained according to the experimental conditions designed by the experimental plan. Obtained results are presented in Table S5.
High values of statistical parameters such as R^2 (square of the correlation coefficient) and Q^2 (cross-validated correlation coefficient) (Q^2: 0.733 and 0.662 for Rs(C/D) and Rs(M/A), respectively and R^2: 0.808 and 0.719 for Rs(C/D) and Rs(M/A), respectively) obtained for created PLS models indicated that models fit well with data and possess high predictive ability. In order to show significance of examined variables, the coefficient plots are displayed in Figure 19.
On these plots the regression coefficients appear as bars and the confidence intervals at 95% confidence limit as error lines. The variable is considered as insignificant if the error line crosses the x-axis and the error is higher than the regression coefficient bar. Coefficients on the bottom side of x-axis have a negative impact on examined output variable, while coefficients on the upper side of x-axis have a positive impact on examined output variable.

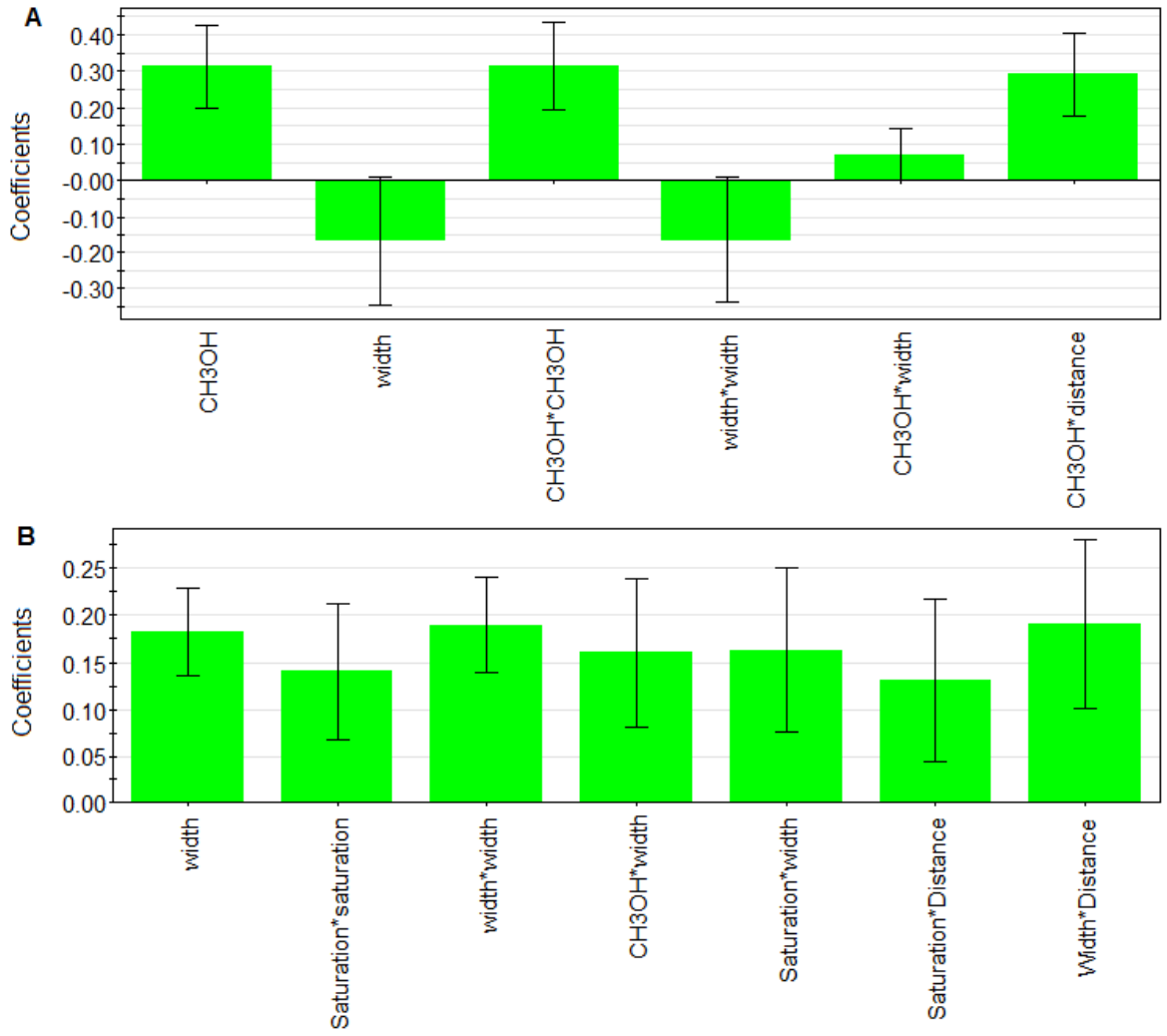


Figure 19. Plot of coefficients for the response variables: (A) Rs (C/D), (B) Rs (M/A).

The coefficients plot for response variable Rs (C/D) (Figure 19A.) indicates that all significant variables such as % of methanol in the mobile phase, CH_3OH x CH_3OH (nonlinear effect) and CH_3OH x distance (interaction effect) are in positive correlation with resolution between impurities C (4-hydroxymoxonidine) and D (6-desmethylmoxonidine), which means that higher values of these variables will result in higher Rs (C/D) values.

Upon examination of the coefficient plot of the Rs (M/A) response variable (Figure 19B.) all significant factors (band width, saturation x saturation (nonlinear effect), width x width (nonlinear effect), methanol x width (interaction effect), saturation x width (interaction effect), saturation x distance (interaction effect), and width x distance (interaction effect)) are in positive correlation with resolution between moxonidine and impurity A (6-chloromoxonidine).

The analysis of coefficients plots and obtained resolution between examined compounds revealed that the best experimental conditions for the separation of moxonidine and its four impurities can be achieved using methanol-toluene-dichloroethane-ammonia 2:3:3:0.1, v/v/v as mobile phase. Under this chromatographic condition resolution between all investigated compounds is satisfied while impurity C (4-hydroxymoxonidine) is moved from the start line and impurity A (6-chloromoxonidine) is not in the front line. Thereby the method specificity was achieved and the migration distances for moxonidine and impurities A (6-chloromoxonidine), B (4-methoxymoxonidine), C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine) were : 58.5 mm ± 0.78 %, 64.4 mm ± 0.95 %, 34.5 mm ± 0.56 %, 16.1 mm ± 0.68 %, and 25.6 mm ± 0.85 %. Retention order of the separated substances (4-hydroxymoxonidine > 6-desmethylmoxonidine > 4-methoxymoxonidine > moxonidine > 6-chloromoxonidine) is basically driven by structural characteristics of the C4/ C6 pyrimidine moiety present in the investigated compounds and by an ability of these compounds to form hydrogen bonds with the siloxane and silanol groups of silica gel. Similar elution order of moxonidine and its impurities was observed in the polar HILIC HPLC system, with one exception only for the reverse order of 6- desmethylmoxonidine and 4-hydroxymoxonidine (6-desmethylmoxonidine > 4-hydroxymoxonidine > 4-methoxymoxonidine > moxonidine > 6-chloromoxonidine). [86]

4.2.2. Method validation

The optimized method was validated according to International Conference on Harmonization (ICH) guidelines with respect to sensitivity, selectivity, linearity, precision, accuracy, and robustness. [37, 38]

4.2.2.1. Estimation of method selectivity

The selectivity of the method was confirmed by observing potential interferences caused by tablet excipients: no interfering peaks were noticed in the chromatogram using the developed chromatographic system, which confirms good selectivity of the method Figure 20.

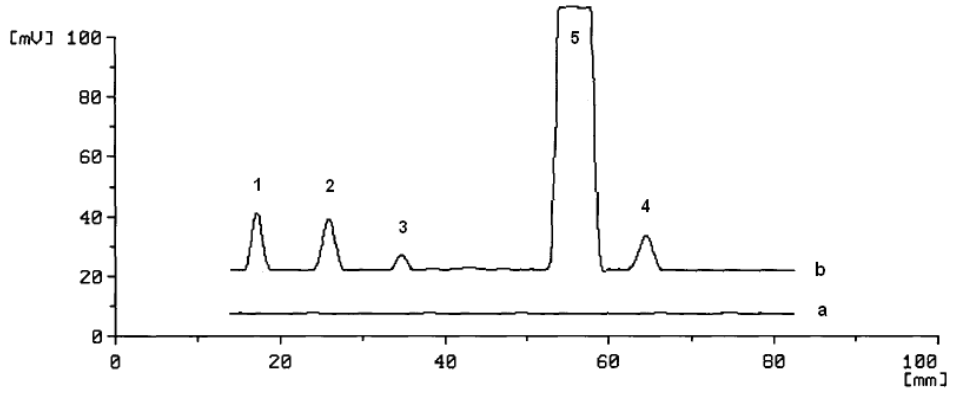


Figure 20. Densitograms obtained for estimation of method selectivity: (a) sample of placebo; (b) standard sample of moxonidine spiked with impurities A:6-chloromoxonidine (4), B:4-methoxymoxonidine (3), C:4-hydroxymoxonidine (1) and D:6-desmethylmoxonidine (2).

4.2.2.2. Examination of method linearity

The linearity was assessed by analysing five working solutions of moxonidine over the concentration range 0.2 to 0.6 mg/mL. This range corresponds to 200-600 ng band^{-1}. Linearity for impurities was investigated from six working solutions ranging from 0.02 to 0.2 mg/mL for impurities A (6-chloromoxonidine), B (4-methoxymoxonidine), C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine) corresponding to 20-200 ng band^{-1}. The relationship between the peak area and the amount of the applied substance was evaluated with use of the linear and the second degree polynomial regression functions. Fitting with the second degree polynomial was done because a wider concentration range is required for quantification of an impurity in the purity method. The high correlation coefficient obtained for all examined compounds ($r > 0.998$) indicated high relationship over the entire concentration range. Calibration curve of examined compounds were presented in Figure 21.

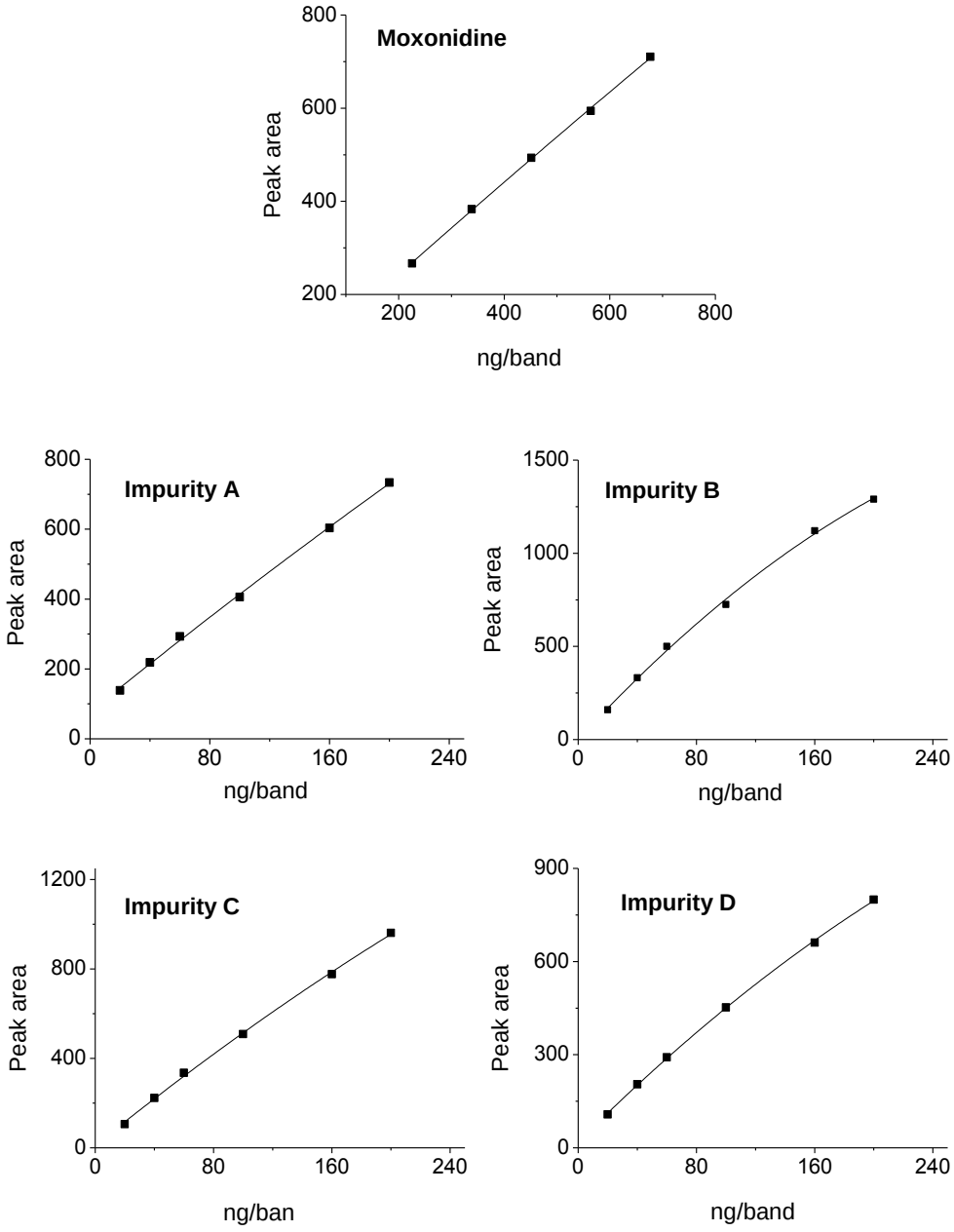


Figure 21. Calibration curves of moxonidine and its impurities (A: 6-chloromoxonidine, B: 4-methoxymoxonidine, C: 4-hydroxymoxonidine, and D: 6-desmethylmoxonidine).

The obtained regression data are summarized in Table 16.

Table 16. Statistical Data for the Calibration Curves-Calibration Function $y = a + bx + x^2$

Compound	Concentration range [ng band^{-1}]	a	b	c	SD	r
Moxonidine	200-600	-22.83 ± 10.67	0.90 ± 0.06	1.24E-04 ± 6.42 E-05	4.306	0.999
Impurity A	20-200	77.66 ± 12.49	3.45 ± 0.29	-9.53E-04 ± 0.001	9.680	0.998
Impurity B	20-200	13.41 ± 32.15	8.06 ± 0.89	-0.007 ± 0.005	21.199	0.998
Impurity C	20-200	10.44 ± 16.86	5.35 ± 0.40	-0.003 ± 0.002	13.062	0.999
Impurity D	20-200	18.77 ± 7.62	4.75 ± 0.18	-0.004 ± 8.09E-04	5.906	0.999

4.2.2.3. Estimation of method accuracy

Accuracy is defined as the degree of agreement between a measured quantity and its true value. It is defines the bias of the method, and the results are expressed as percentage recovery. [37] To avoid systematic errors, an effect of larger amounts of moxonidine on the peak shape and resolution of impurities had to be tested. Method accuracy was therefore verified by determination of impurities A (6-chloromoxonidine), B (4-methoxymoxonidine), C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine) in the presence of moxonidine. The laboratory-made placebos were spiked with moxonidine and a mixture of 0.3 %, 0.5 %, and 1.2 % impurities 6-chloromoxonidine, 4-methoxymoxonidine, 4-hydroxymoxonidine, and 6-desmethylmoxonidine, respectively. The application volumes were 40 and 20 µl for the estimation of 0.3 %, 0.5 %, and 1.2 % impurities, respectively. The solutions were made in triplicate. Scanned profiles obtained for the moxonidine samples spiked with impurities are presented in Figure 22.

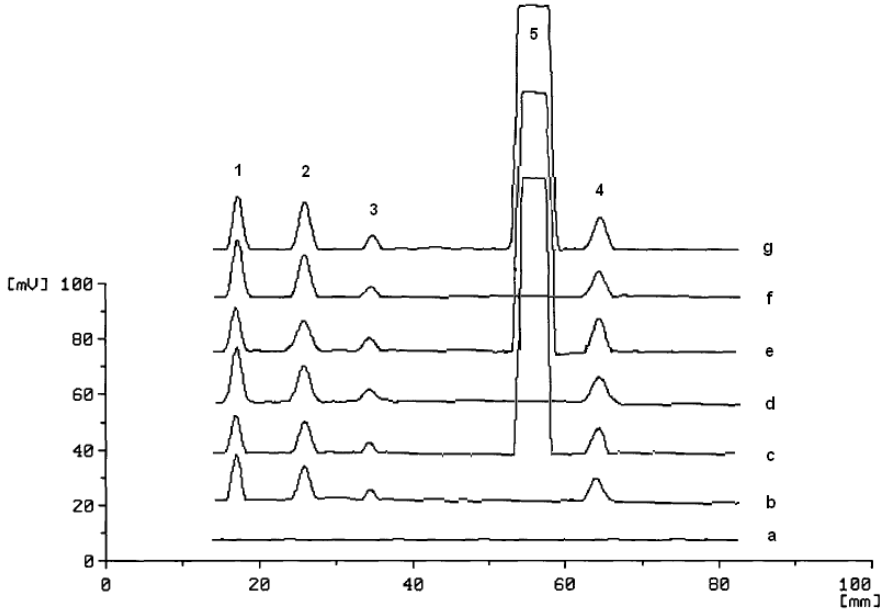


Figure 22. Densitograms obtained at the wavelength 280 nm for (a) sample of placebo; (b, d, f) standards of impurities A: 6-chloromoxonidine (4), B: 4-methoxymoxonidine (3), C: 4-hydroxymoxonidine (1) and D: 6-desmethylmoxonidine (2) at 0.3 %, 0.5 % and 1.2 % level, respectively; (c, e, g) placebo spiked with moxonidine and impurities 6-chloromoxonidine, 4-methoxymoxonidine , 4-hydroxymoxonidine and 6-desmethylmoxonidine at 0.3 %, 0.5 % and 1.2 % level (5, 4, 3, 1 and 2, respectively).

Calculated recoveries were plotted against the expected values (corresponding to the standards without moxonidine). The obtained recovery and standard deviation (RSD) values were satisfactory and meet the requirements for the method accuracy (95%-105% for active ingredients, 70.0-130.0% for impurities A (6-chloromoxonidine) and B (4-methoxymoxonidine) (0.1% <x<0.5%), or 80.0-120.0% for impurities C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine) (0.5% <x <1.0%)). [131] Results are presented in Table 17.

Table 17. Accuracy of the method

Compound	Level [%]	Mean Recovery [%]	RSD [%]
Moxonidine	80	99.52	1.38
	100	100.36	0.89
	120	99.81	0.95
Impurity A	0.3	103.28	5.89
	0.5	107.63	2.20
	1.2	101.07	2.37
Impurity B	0.3	98.29	2.48
	0.5	104.40	4.25
	1.2	97.87	2.06
Impurity C	0.3	100.37	2.96
	0.5	93.95	3.84
	1.2	90.10	3.87
Impurity D	0.3	95.84	1.78
	0.5	101.76	3.76
	1.2	95.66	2.07

4.2.2.4. Estimation of method precision

The precision of the method was studied as repeatability of the system which was evaluated by six repetitive measurments (n=6) of moxonidine and impurities A (6-chloromoxonidine), B (4-methoxymoxonidine), C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine) at three different concentrations levels and the results were expressed in terms of relative standard deviation (RSD %). The obtained values are presented in Table 18 and fulfilled the required criteria [RSD 2% for the active substance, 10% for impurities 4-hydroxymoxonidine, and 6-desmethylmoxonidine, and 15% for impurities 6-chloromoxonidine, 4-methoxymoxonidine. [131]

Table 18. Precision of the method (n=6)

Amount [ng band^{-1}]	Moxonidine RSD [%]	Impurity A RSD [%]	Impurity B RSD [%]	Impurity C RSD [%]	Impurity D RSD [%]
200	1.00				
400	0.74				
600	0.68				
50		2.32	3.89	3.74	2.71
80		1.40	2.63	1.44	1.75
190		1.81	1.50	1.29	1.19

4.2.2.5. Estimation of method sensitivity

The sensitivity of the method was investigated by calculating the limit of detection (LOD) and limit of quantification (LOQ). LOD is the lowest quantity which can be detected but not necessary with suitable precision while LOQ is the lowest quantity which can be determined with suitable precision The LOD and LOQ values were obtained experimentally and statistically. Experimentally obtained LOD values for impurities A (6-chloromoxonidine), B (4-methoxymoxonidine), C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine) were equal to 7 ng per band, while experimentally obtained LOQ values were equal to 20 ng per band (corresponding with the impurity levels of 0.04 % and 0.12 % respectively). Statistically, the LOD values were determined by fitting the inter-day back-calculated standard deviation for each calibration standard. The y-

intercept was then equal to SD_0 (the estimated standard deviation at zero concentration). LOD was defined as $3SD_0$ and LOQ as $10SD_0$. The LOD values obtained for impurities 6-chloromoxonidine, 4-methoxymoxonidine, 4-hydroxymoxonidine, and 6-desmethylmoxonidine were 8.41, 7.89, 7.32, and 3.73 ng, respectively (equivalent to the impurity levels of 0.053 %, 0.049 %, 0.046 %, and 0.023 % respectively). The LOQ values for impurities 6-chloromoxonidine, 4-methoxymoxonidine, 4-hydroxymoxonidine, and 6-desmethylmoxonidine were 28.00, 26.30, 24.41, and 12.43 ng, respectively (equivalent to the impurity levels of 0.175 %, 0.164 %, 0.153 %, and 0.078 %, respectively).

4.2.2.6. Estimation of robustness test

According to the ICH [38] regulations, the evaluation of robustness should be considered during the method development phase and it should reflect the reliability of an analytical procedure with respect to deliberate variation in method parameters. Robustness is a measure of the capacity of the method to remain unaffected by small yet deliberate variations of working condition, and it is indicative of the method reliability. ICH guideline provide some recommendation for the factors that should be examined during robustness testing. In this study, the robustness test includes the influence of the variations of the following parameters: variations of different amounts of methanol in mobile phase (24.7 ± 1 %), variation of different developing distances (90 ± 2 mm), different band width (10 ± 0.5 mm), saturation time (15 ± 2 min) and different chamber geometry (twin-trough and flat). Selection of the tested factors was based on our experience and observations made in the course of method development. All experiments were performed applying one-factor-at-a-time approach, which means that one factor was changed while others were kept on constant level. The developed method showed to be significantly robust regarding to resolution factor between moxonidine and its impurities.

4.2.3. Determination of moxonidine and its impurities in dosage form

The method was used to screen the commercial dosage forms (Moxogamma® 0.4 mg tablet). According to the health authorities e.g FDA and EU, the allowed content of moxonidine and its impurities to meet the requirments for manufacture in the commercially available pharmaceutical dosage form is in a range of 95%-105% for

moxonidine, and for impurities A (6-chloromoxonidine), and B (4-methoxymoxonidine) below 0.5% while impurities C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine) below 1%. The densitogram obtained for moxonidine and its impurities is shown in Figure 23.

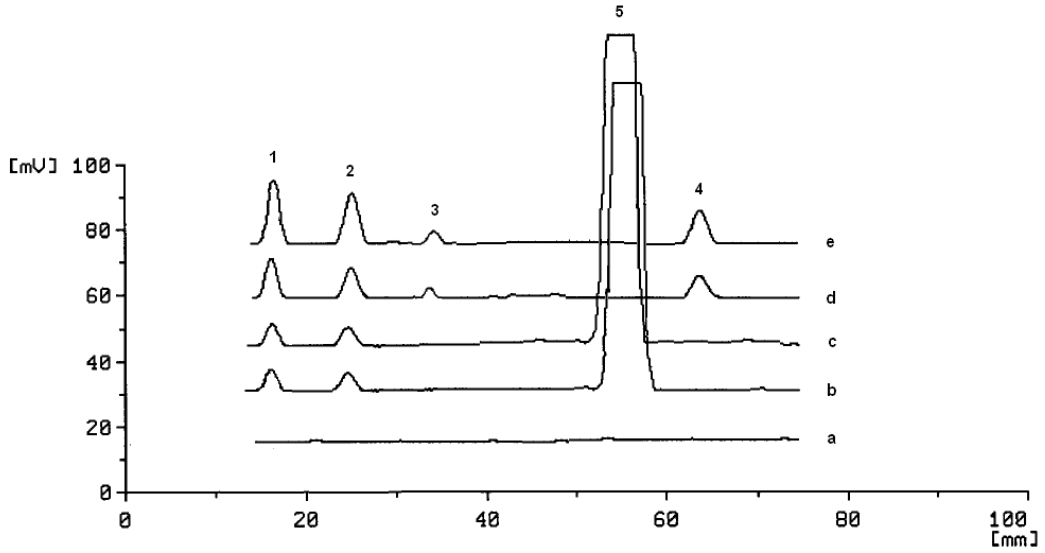


Figure 23. Densitogram obtained at the wavelength 280 nm for (a) sample of placebo; (b, c) samples of the moxonidine tablet; (d) standards of impurities A: 6-chloromoxonidine, B: 4-methoxymoxonidine, C: 4-hydroxymoxonidine and D: 6-desmethylmoxonidine at 0.3 % level (4, 3, 1 and 2, respectively); (e) standards of impurities 6-chloromoxonidine, 4-methoxymoxonidine, 4-hydroxymoxonidine and 6-desmethylmoxonidine at 0.5 % level (4, 3, 1 and 2, respectively).

The results obtained for the content of moxonidine of 97.41 % and the found levels of impurities C (4-hydroxymoxonidine), and D (6-desmethylmoxonidine) of 0.21% and 0.26 %, respectively, meet the requirements of the manufacture, and do not exceeded 1.0 %. The contents of impurities A (6-chloromoxonidine) and B (methoxymoxonidine) were estabilished as lower than LOD of the proposed method and meet the requirements of the manufacture, and do not exceeded 0.5 % (Table 19).

Table 19. Assay of moxonidine and its impurities

Sample	Moxonidine [% ± RSD]	Impurity A [% ± RSD]	Impurity B [% ± RSD]	Impurity C [% ± RSD]	Impurity D [% ± RSD]
Moxogamma® 0.4 mg	97.41 ± 1.92	n.d.	n.d.	0.21 ± 6.87	0.26 ± 4.82

5. Conclusion

The obtained results lead us to these conclusions:

1. The retention behavior and lipophilicity of 16 alpha adrenergic and imidazoline receptor ligands was investigated using three different chromatographic systems: methanol-water/RP-18, tetrahydrofuran-ammonia-water/RP-18, and tetrahydrofuran-ammonia-water/CN and different mode of development (horizontal and vertical).

- In all instances chromatographic behavior of the investigated substances is in accordance with reversed-phase condition i.e. retention increase with increasing polarity of the solvent system used.
- Despite to the established linear relationships between R_M^0 and m, as well as R_M^0 and C_0 values (except for the methanol-water/RP-18 system) the correlation of chromatographic indices with calculated log *P* values revealed that the most reliable parameter for assessment of lipophilicity of I-IR/α-AR ligands is R_M^0 value while C_0, and m values are less reliable.
- High correlations obtained between the ACD/log *P* and R_M^0 values in the tetrahydrofuran-ammonia-water/CN system (r = 0.933), and the ACD/log *P* and R_M^0 values in the tetrahydrofuran-ammonia-water/RP-18 system (r = 0.916) point out to these systems as suitable candidates for the estimation of lipohilicity of the tested compounds.
- Performed PCA analysis used for examination of similarity and differences in lipophilicity between the tested compounds showed that according to experimentally determined (R_M^0) and calculated lipophilicity (log *P*) parameters, 16 examined compounds can be classified into four different lipophilicity groups.

2. The QSRR modeling of 16 alpha adrenergic and imidazoline receptors ligands was performed with the use of partial least square regression, in order to select the most important variables that describe the behavior of the investigated compounds.

- The best statistical parameters were obtained for QSRR (THF-NH_3-H_2O/RP-18) model, while the lowest power for prediction of the test set showed QSRR (CH_3OH-H_2O/RP-18) model.

- In addition to the logarithm of the partition coefficient selected in all devised QSRR models, the average molecular weight (AMW), P_VSA-like on van der Waals volume, bin 3 (P_VSA_v_3) descriptors and hydrogen bonding properties of the tested compounds are the most relevant descriptors influencing the retention behavior in the RP-TLC systems.
- Obtained chromatographic data proved to be reliable parameters for describing the lipophilic properties of the investigated compounds, containing more valuable information than calculated lipophilicity values.

3. New, simple and reliable TLC method was developed and validated for simultaneous determination of moxonidine and its four impurities.

- The proposed TLC method fulfilled all validation requirments according to International conference on harmonization guidelines and obtained results showed that content of moxonidine and its four impurities meet the requirements of manufacturers.

6. Literature

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7. Supplementary materials

Supplement A: List of tables

Table S1. C_0 values calculated for the examined systems

Compound	CH_3OH-H_2O/RP-18	THF-NH_3-H_2O/RP-18	THF-NH_3-H_2O/CN
	C_0	C_0	C_0
Moxonidine	0.773	0.363	0.188
Brimonidine	1.000	0.338	0.249
Tizanidine	0.835	0.609	0.515
Clonidine	0.868	0.635	0.552
Amiloride	1.051	0.436	0.501
Guanfacine	1.175	0.596	0.490
Guanabenz	0.972	0.629	0.540
Idazoxan	0.855	0.642	0.538
Efaroxan	0.819	0.675	0.586
Harmine	1.054	0.632	0.537
Harmane	1.005	0.640	0.550
Naphazoline	0.964	0.772	0.753
Tetrahydrozoline	0.838	0.798	0.817
Tramazoline	0.848	0.853	0.833
Oxymetazoline	0.936	0.786	0.773
Xylometazoline	0.907	0.820	0.812

Table S2. Correlation matrix of log *P* values

	Alog P_s	AClog *P*	milog *P*	Alog *P*	Mlog *P*	KOWWIN	Xlog *P2*	Xlog *P3*	Clog *P*	ACD/log *P*	ChemAxon Log *P*
Alog P_s	1.000										
AClog *P*	0.869	1.000									
milog *P*	0.947	0.909	1.000								
Alog *P*	0.847	**0.961**	0.922	1.000							
Mlog *P*	0.731	0.690	0.780	0.805	1.000						
KOWWIN	0.905	0.736	0.895	0.766	0.769	1.000					
Xlog *P2*	0.790	0.833	0.903	0.895	0.800	0.733	1.000				
Xlog *P3*	0.838	0.834	0.816	0.781	**0.512**	0.701	0.763	1.000			
Clog *P*	0.911	0.800	0.924	0.805	0.742	0.928	0.822	0.736	1.000		
ACD/log *P*	0.852	0.784	0.903	0.802	0.658	0.909	0.814	0.725	0.901	1.000	
ChemAxon Log *P*	0.916	0.845	0.948	0.898	0.878	0.929	0.873	0.735	0.947	0.873	1,000

Table S3. Correlations between retention parameters (R_M^0, *m* and C_0) and calculated log *P* values for CH_3OH-H_2O/RP-18 chromatographic system

	R_M^0	m	C_0
Alog P_s	0.402	-0.300	0.020
AClog *P*	0.310	-0.287	-0.158
milog *P*	0.398	-0.297	-0.012
Alog *P*	0.216	-0.219	-0.154
Mlog *P*	0.102	-0.169	-0.205
KOWWIN	0.405	-0.347	-0.105
Xlog *P2*	0.340	-0.200	0.074
Xlog *P3*	0.465	-0.234	0.246
Clog *P*	0.580	-0.507	-0.136
ACD/log *P*	0.536	-0.450	-0.126
ChemAxon Log *P*	0.369	-0.347	-0.148

Table S4. The descriptor values employed in the selected QSRR models

ID	**Training set**	**ACD/log *P***	**Clog *P***	**nON**	**nN**	**P_VSA_e_2**	**P_VSA_v_3**	**AMW**
1	Moxonidine	0.325	1.308	6	5	38.177	77.326	8.370
2	Brimonidine	1.202	1.508	5	5	50.195	96.149	10.470
4	Clonidine	2.362	1.428	3	3	43.072	121.369	9.630
5	Amiloride	0.925	0.108	8	7	13.309	52.458	9.985
7	Guanabenz	2.662	2.979	4	4	47.112	125.409	10.092
8	Idazoxan	2.307	1.814	4	2	58.298	58.298	7.331
9	Efaroxan	3.098	2.842	3	2	65.542	65.542	6.585
11	Harmane	3.060	3.058	2	2	76.220	76.220	7.330
12	Naphazoline	2.988	3.826	2	2	83.739	83.739	6.816
14	Tramazoline	2.782	2.485	3	3	52.580	52.580	6.363
15	Oxymetazoline	3.764	4.609	3	2	78.960	74.193	5.942
16	Xylometazoline	4.592	5.376	2	2	83.669	83.669	5.708
	Test set							
3	Tizanidine	2.385	2.135	5	5	64.781	103.929	10.190
6	Guanfacine	1.330	1.370	4	3	45.812	124.110	9.885
10	Harmine	3.054	3.129	3	2	82.870	82.870	7.354
13	Tetrahydrozoline	2.845	3.535	2	2	58.419	58.419	6.291

Table S5. Experimental plan and obtained results

Experiment No.	CH3OH (%)	Saturation (min)	width (mm)	distance (mm)	Rs(M/A)	Rs(C/D)
1	24.7	15	8	180	2.757	3.321
2	19.7	5	8	160	2.259	2.258
3	19.7	5	8	200	2.323	2.749
4	29.7	5	8	200	2.900	4.430
5	19.7	25	8	160	3.165	2.367
6	19.7	25	8	200	3.851	2.403
7	29.7	25	8	160	3.835	4.389
8	24.7	15	10	180	3.041	2.888
9	24.7	15	10	180	2.902	2.937
10	24.7	15	10	180	2.840	3.181
11	24.7	15	10	180	2.487	3.075
12	24.7	15	10	160	2.806	2.593
13	24.7	15	10	200	2.841	2.645
14	19.7	15	10	180	3.416	1.907
15	29.7	15	10	180	3.667	4.020
16	24.7	5	10	180	2.840	3.070
17	24.7	15	10	180	2.607	3.470
18	24.7	25	10	180	4.362	3.585
19	24.7	15	12	180	3.985	2.762
20	19.7	5	12	160	4.163	2.642
21	29.7	5	12	160	4.458	3.212
22	29.7	5	12	200	4.944	3.787
23	19.7	25	12	200	6.267	2.244
24	29.7	25	12	200	6.583	3.729
25	29.7	25	12	160	4.843	3.324

Supplement B: List of published papers

-Article published in international scientific journal (M23)

1. M.S.M. Shenger, S. Filipic, K. Nikolic, D. Agbaba, Estimation of lipophilicity and retention behaviour of some alpha adrenergic and imidazoline receptor ligands using reversed phase-thin layer chromatography. Journal of Liquid Chromatography & Related Technologies 37 (2014) 2829-2845.

2. M.S.M. Shenger, S. Filipic, K. Nikolic, D. Agbaba, Determination of moxonidine and its impurities by thin layer chromatography. Journal of Liquid Chromatography & Related Technologies 38 (2015) 1121-1125.

- Proceedings on international congresses (M33)

S. Filipic, M.S.M. Shenger, J. Vucicevic, M. Popovic, D. Agbaba, RP-TLC in Quantitative Structure-Retention Relationships of some alpha adrenergic and imidazoline receptor ligands. 12th International Conference on Fundamental and Applied Aspects of Physical Chemistry, 22-26 Septembar 2014, Belgrade, Serbia.

- Abstract on international congresses (M34)

1. M.S.M. Shenger, S. Filipic, K. Nikolic, D. Agbaba. Determination of moxonidine and its impurities by thin layer chromatography. VI Serbian Congress of Pharmacy with international participation, October 15-19, 2014, Belgrade, Serbia.

2. S. Filipic, M.S.M. Shenger, K. Nikolic, D. Agbaba. Optimization of chromatographic condition for separation of moxonidine and its impurities by TLC. 3rd congress of pharmacists' of Bosnia and Herzegovina with international participation. May 14-17, 2015, Sarajevo, Bosnia i Herzegovina.

8. Biography

Musbah Salem Mohamed Shenger was born on 15.8.1968. In Tripoli, Libya. He completed secondary school in 1987 and started University study at Faculty of Pharmacy, University of Tripoli in academic 1987/1988 year. He graduated from the Faculty of Pharmacy in 1993/1994. From 1996 to 2009 he was worked in Tripoli in his own private Pharmacy. In October 2009 he continued academic studies in Serbia and in 2009/2010 obtained the master degree from the University of Belgrade - Faculty of Chemistry, Department of Analytical Chemistry, where he defended his master's thesis entitled: "Determination of an ions in some pharmaceutical preparation". Since 2011/2012 academic year he has been enrolled in PhD study at the Department of Pharmaceutical Chemistry, University of Belgrade - Faculty of Pharmacy under the supervision of Professor Danica Agbaba and Docent Slavica Oljačić.

Musbah Salem Mohamed Shenger has two published articles in international scientific journals, category M23, one proceeding on international congresse (M33) and two abstracts on international congresses (M34).

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Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Квантитативни односи структуре и ретенционих особина одабраних лиганада алфа адренергичких и имидазолинских рецептора у танкослојној хроматографији

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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(Молимо да заокружите само једну од шест понуђених лиценци.
Кратак опис лиценци је саставни део ове изјаве).

Потпис аутора

У Београду, 18.01.2017

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