Chromatographic Techniques in Pharmaceutical Analysis

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High-performance liquid chromatography (HPLC) is an incredibly universal tool, especially when combined with different detectors, such as UV-Visible spectroscopy, mass spectrometry (MS), and fluorescence detection for numerous active ingredients in different pharmaceutical formulations without interferences from other excipients. TLC, in combination with densitometry, is a very efficient tool for the determination of biologically active substances present in pharmaceutical preparations. In addition, TLC coupled to densitometry and mass spectrometry could be suitable for preliminary screening and determination of the biological activity (e.g., antioxidant properties, thin layer chromatography (TLC) by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method) of plant materials. Gas chromatography, coupled with a mass spectrometer (GC-MS, GC-MS/MS), is of particular importance in the testing of any volatile substances, such as essential oils. LC, coupled to NMR and MS, is the best solution for identifying and studying the structure of unknown components from plant extracts, as well as degradation products (DPs). Thanks to size-exclusion chromatography, coupled to multi-angle light scattering, the quality control of biological pharmaceuticals is possible.

pharmaceuticals chromatographic techniques liquid chromatography gas chromatography thin layer chromatography

1. Introduction

All pharmaceuticals can be classified as natural or synthetic bioactive compounds. It should be emphasized that there is no uniform definition of a bioactive substance in the scientific literature [1]. Because of a wide pharmacological spectrum of natural and synthetic bioactive compounds, such as anti-cancer properties, their role in the design of new drugs and more effective pharmaceutical formulations applicable in the treatment of many diseases is also important. Natural and synthetic bioactive compounds are used in the production of pharmaceuticals, nutraceuticals, dietary supplements and cosmetics. A very important element of chemical analysis is testing the purity and quantification of a biologically active substance of natural or synthetic origin in pharmaceutical preparations [2][3][4][5][6][7]. Chromatographic methods are an excellent tool in the analysis of bioactive compounds present in pharmaceutical preparations and extracted from plant medicinal substances. Both planar techniques, as well as high performance liquid chromatography and gas chromatography, allow the systematic analysis of the qualitative and quantitative composition and the comparison of individual plant substances based on differences and similarities in their composition. Chromatography is a technique of separating multicomponent mixtures into single components, which are the result of their different migration speeds along the

bed of the stationary phase. Unlike other classical separation techniques (such as crystallization, extraction or distillation), it allows for the separation of compounds without the need for in-depth information about the type of substances to be separated, i.e., their number and their relative amounts in the mixture [8][9]. The versatility and resolving power of chromatographic systems has made chromatography an excellent analytical technique intensively used for scientific, industrial and medical purposes. It is not only a key element of the methodology of many scientific studies, but also a tool for monitoring the state of the environment or the production of pure compounds in the pharmaceutical and chemical industries. The greatest application of chromatography is in the analysis of pharmaceuticals (30%), biochemical and clinical chemistry (25%), environmental protection (15%), food and cosmetics (10%), inorganic substances (5%); analyses in other fields accounts for 15% [10]. In turn, chromatography is used, on average, in 75% of the total number of analyses developed in various pharmacopoeias.

2. Thin Layer Chromatography

The natural and synthetic bioactive compounds were analyzed by TLC technique. Mainly synthetic medicinal substances in pharmaceutical preparations, e.g., antidiabetic drugs (metformin, saxagliptin, dapaglifloxin, linagliptin), antibiotics (gatifloxacin, ofloxacin, cefoperazone, chloramphenicol), steroid compounds (dexamethasone, prednisone, clobetasol, estradiol norethisterone, estrone, estriol, testosterone), vitamins (D, and K), alkaloids, non-steroidal anti-inflammatory drugs belonging to different groups (ibuprofen, meloxicam, celecoxib, etoricoxib, firecoxib, robenacoxib and cimicoxib), and antihypertensive compounds (reserpine, lupeol, scopoletine, bacoside A, piperine in *Cardimap tablet*) were investigated. Herbal medicine or phytotherapy is a type of treatment in which raw materials and plant preparations are used. Therefore, the subject of research was natural bioactive compounds found mainly in plants, such as steroidal (diosgenin, stigmasterol, ecdysteroids, β-sitosterol) and phenolic compounds (gallic acid), anticancer substances (arctiin, arctigenin), tannins, saponins, coumarins, alkaloids (trigonelline, berberine, barbamine, palmitine, magnoflorine, jatrorrhizine, ephedrine), flavonoids (i.a. diosmin, rutin), triterpenes (betulinic acid), essential oils, and antidepressants (salidroside, rosavin, p-tyrosol, and hydroquinone).

Parys et al. [11] used the TLC-densitometric technique for identification of meloxicam, as well as non-steroidal anti-inflammatory drugs. Different kinds of chromatographic plates were applied, such as: neutral aluminum oxide $60F_{254}$ and $150F_{254}$, silica gel 60 and $60F_{254}$, mixture of silica gel 60 and Kieselguhr $60F_{254}$, silica gel RP-$18F_{254}$ and RP-$8F_{254}$. The mixtures of ethyl acetate-toluene-*n*-butylamine (2:2:1, *v*/*v*/*v*) and ethyl acetate-ethanol-toluene-25% NH_4OH (6:3:1:0.06, *v*/*v*/*v*/*v*) were used in the NP-TLC analysis. For the RP-TLC analysis, a mixture of methanol-water (5:5, 8:2, *v*/*v*) was applied. Processes of spot visualization of meloxicam were carried out by the use of various visualization agents, such as: rhodamine B, Janus blue, methyl green, brilliant green, crystalline violet, alkaline blue, gentian violet, methylene violet, fuchsine and brilliant cresyl blue. Among all chromatographic conditions studied, the best proved to be silica gel $60F_{254}$ and neutral aluminum oxide plates, as well as the mixture of ethyl acetate-toluene-*n*-butylamine (2:2:1, *v*/*v*/*v*) in NP-TLC. RP-$8F_{254}$ and RP-$18F_{254}$ plates and the mixture of methanol-water (8:2, *v*/*v*) enabled detection of meloxicam at the lowest concentration level in RP-TLC.

Moreover, of all visualization agents studied, the smallest limit of detection of meloxicam provided crystalline violet and gentian violet as new visualization agents on silica gel $60F_{254}$ and neutral aluminum oxide $150F_{254}$, respectively.

The paper published by Megawati and coworkers [12] indicate the applicability of thin-layer chromatography for the separation, identification and bioactivity i.e., antioxidant activity as well as toxicity of selected steroids, as the new renewable bioactive compounds obtained from plant material, such as algae cultivated in Indonesia. The steroids extracted from red algae *Eucheuma cottonii* and *Chlorella sp.* coming from Indonesia have been separated on a silica gel plate F_{254} activated by heating in an oven by using the eluent *n*-hexane-ethyl acetate in a proper volume composition and next identified under UV at 254 and 366 nm, as well as by using Liebermann–Burchard reagent [12].

3. Column Liquid Chromatography

High Performance liquid chromatography (HPLC) is the dominant technique in pharmaceutical and phytochemical analysis of various bioactive compounds. Compared with other analytical techniques, HPLC offers high sensitivity and accuracy of qualitative and quantitative analysis of pharmaceutically active compounds (APIs) in different samples (matrices), such as synthetic drug products and plant materials [13][14][15].

Newly published papers indicate that RP-HPLC could simultaneously detect in one HPLC run various NSAIDs in combined pharmaceutical formulations [16][17][18][19]. For instance, an RP-HPLC method has been used as a stability-indicating assay for active ingredients, namely indomethacin, paracetamol, famotidine and the newly synthesized codrug (indomethacin-paracetamol) [16]. Another work prepared by Al-Khateeb and coauthors shows a simple, rapid, efficient and green method of high-temperature liquid chromatography (HTLC) for the separation and simultaneous determination of selected NSAIDs (ketoprofen, naproxen, sodium diclofenac, ibuprofen) in pharmaceuticals, as well of NSAIDs trace in water samples [17]. Growing research on the simultaneous analysis of the combination of NSAIDs, i.e., aspirin, paracetamol, and naproxen by isocratic RP-HPLC method using short column also confirms the next two papers published by Hassan and Nam [18][19]. Reported HPLC methods are cost-effective way to determine NSAIDs in a combined mixture.

An RP-HPLC technique was also applicable as a stability-indicating method for the analysis of active ingredients and their impurities in pharmaceutical formulations of some drugs affecting the autonomic nerves system, such as ephedrine in the presence of naphazoline and neostigmine methylsulfate [20][21]. The RP-HPLC analysis proposed by Parab and coworkers allows for the simultaneous quality control, forced degradation and stability study of neostigmine methylsulfate in bulk drug and injection formulation [21].

HPLC-MS/MS profiling of potential antioxidants i.e., some phenolic acids and flavonoids in extracts of *Salvia* species from Jordan have been successfully presented by Al-Jaber and coworkers [22]. The results obtained confirmed that the three *Salvia* species exhibited good antioxidant activity due the presence of four bioactive

phenolic acids, including gallic acid, caffeic acid, rosmarinic acid and salvianolic acid B and seven other flavonoids, such as luteolin-7-O-glucoside, apigenin, apigenin-7-O-glucoside, rutin, nariginin, hesperidin and quercetin [22].

In the case of the HPLC analysis combined to SEC-MALS, a mixture containing phosphate buffered saline (PBS) as mobile phase and SEC column, i.e., silica-based or polymers-based are usually used. For instance, Fu et al. used this technique in a quality study of recombinant anti-interleukin-23 monoclonal antibodies as potential anticancer drug candidates [23]. Another work shows utility of size-exclusion chromatography in purity control of next antibody-drug conjugates (ADCs) as anticancer agents. In this work, a novel approach to derive homogeneous ADCs with a drug-to-antibody ratio of 2 from any human immunoglobulin 1 (IgG1), using trastuzumab as a model, was described [24]. A similar study was presented by Farras and coworkers concerning the search of functional monoclonal antibodies (mAbs) based on trastuzumab structure [25]. Another paper shows that HPLC combined to SEC-MALS was a fast method for characterizing the content of capsids in samples containing adeno-associated virus (AAV) and, thus, for supporting the development of therapeutic AAV-based drugs [26].

4. RP-TLC/RP-HPLC and Lipophilicity

Morak-Młodawska et al. [27] investigated the lipophilic properties of 1,2,3-triazole-dipyridothiazine with antitumor activity by the RP-TLC method. The lipophilicity parameters obtained by the TLC technique were compared with the logP obtained by calculation. They found that the calculation methods did not give good approximations to the values of the lipophilicity parameters obtained experimentally with the RP-TLC technique. Another work presents the application of chromatographic parameters R_F and next R_M to determine the lipophilicity descriptor according to the Soczewiński–Wachtmeister methodology for twenty-one newly synthesized chalcones [28]. Thin-layer chromatography analysis was performed using reversed phase silica gel 60 RP-18F_{254} plates (RP-TLC) and three binary phases: acetonitrile–water, ethanol–water and acetone–water. Among all mobile phases used, the best was that which consisted of acetonitrile-water. This mixture allowed one to predict the most reliable chromatographic parameter of lipophilicity (C_0) best correlated with calculated logP. In addition to this, the performed QSRR analysis (quantitative structure-retention relationship) between C_0 and selected molecular descriptors was helpful to determine those with the highest influence on C_0, and, thus, on the lipophilicty parameter of examined compounds. Thin layer chromatography and the Soczewiński–Wachtmeister method were also used for the evaluation of the lipophilicity of spirohydantoins applied in the treatment of epilepsy and diabetes [29]. High correlations were stated between lipohilicity and plasma protein binding and between lipophilicity and the blood–brain barrier [29]. Dołowy et al. [30] investigated by NPTLC and RPTLC techniques the lipophilicity of antidiabetic drugs, namely metformin and phenformin. The obtained results of lipophilicity were compared with the theoretical values of the partition coefficients of the studied drugs. They showed difficulties in obtaining reliable lipophilicity values. They indicated that theoretical methods based on the Gutman and Randic topological indexes could be used as new procedures to calculate the lipophilicity of metformin and phenformin.

5. Gas Chromatography

The recently published papers indicate that gas chromatography (GC) with different detection systems, such as flame-ionization detector (GC-FID), or coupled to single or tandem mass spectrometric approaches (GC-MS, GC-MS/MS), served as an effective tool for the determination of various bioactive compounds in pharmaceutical and phytochemical analysis [31][32][33][34][35][36][37][38][39][40][41][42][43][44][45][46][47][48][49][50][51][52][53][54][55].

GC is a useful technique for testing any volatile substances, such as essential oils, which are a promising group of natural products of the aromatic plants, as a result of their various biological effects, i.e., their antioxidant, antibacterial, antimicrobial, and antitumor activities [38][39][40][41][42][43][44][45].

The next papers [33][34][35][36][37] show the applicability of GC analysis for the determination of impurities in drugs. For example, GC-FID was successfully applied to the determination of methyl, ethyl and isopropyl methanesulfonates impurities (MMS, EMS, IPMS) in the delgocitinib drug [33]. Analysis were performed on the DB-624 UI column (30 m × 0.25 mm × 1.4 µm), using helium as the carrier gas with a flow rate of 5.0 mL/min. The developed method showed good linearity, accuracy and precision from 1 to 5 ppm. The LOD and LOQ were 0.1 and 0.3 ppm for MMS, 0.05 and 0.1 ppm for EMS and IPMS, respectively [33]. Ahirrao et al. [34] used the GC-MS/MS method for the estimation of genotoxic impurities, i.e., methyl methane sulfonate (MMS), ethyl methane sulfonate (EMS) and isopropyl methane sulfonate (IMS) in alalevonadifloxacin mesylate (ALA), which is a new antibiotic approved in India to treat infections caused by Gram-positive bacteria. The study was conducted using the Rtx-624 column (30 m × 0.32 mm × 1.8 µm) and helium as the carrier gas, with a flow rate equal to 1.5 mL/min. The proposed method was highly sensitive with LOQ for MMS, EMS and IMS as 5, 10, and 20 ng/g of ALA, respectively [34].

In another paper, the GC-MS analysis was applied to the determination of the phytocomponents present in *Clerodendrum serratum Linn* roots, used in the treatment of various diseases, such as cough, asthma, rheumatism fever and cephalalgia ophthalmia in the Ayurveda and Siddha medicine in India [53]. Next, the GC-FID method was used for the determination of squalene, which was one of the constituents present in *C. serratum Linn* roots. The GC-MS measurements were performed on the Elite-5MS column (30 m × 0.25 mm × 1 µm), with helium as the carrier gas. Whereas, the GC-FID measurements were performed on the Zebron DB column (30 m × 0.25 mm × 0.50 µm), with nitrogen as the carrier gas with flow rate 12.7 mL/min. The obtained results showed that squalene, methyl palmitate, hexadecenoic acid and stigmasterol were major constituents in *C. serratum Linn* roots. Intra- and interday precision of squalene ranged from 1.154 ÷ 1.819% and 1.5102 ÷ 1.861%, respectively. The LOD and LOQ of analyzed squalene were 0.79 µg/mL and 1.89 µg/mL, respectively [53].

6. Combined Techniques

The latest trends in the determination of bioactive compounds present in various complex matrices concern the so-called combined techniques (coupled techniques). In these techniques, separation methods are combined with various detection methods. Chromatographic methods (e.g., LC, GC) are mainly used as separation techniques, while spectroscopic methods (MS, NMR) are used as detection methods. Combined techniques also include combinations of several chromatographic methods. The combined technique should be selective for the analytes to

be determined, be sensitive over a wide range of concentrations, and should enable the best possible identification of the substances to be determined. The nature of the analyte, the ease of combining the various methods, the sensitivity of the determinations required and the availability of the equipment should determine the choice of the appropriate combined technique. Numerous papers reported the utility of combined techniques in pharmaceutical and phytochemical analysis [56][57][58][59][60][61][62][63][64][65].

High performance thin layer chromatography and high performance liquid chromatography were used for the analysis of flavonoids and phenolic acids found in *Flos Lamii albi* [56]. HPTLC analyses were performed on Si $60F_{254}$ plates using a multiple gradient development program. For the analysis of flavonoids at a distance of 3 cm, the following were used: 6 mL solution A (toluene: hexane: formic acid, 7: 3: 0.1), 3 mL ethyl acetate, and 1 mL methanol. Then, 5 mL solution A (toluene: hexane: formic acid, 7: 3: 0.1), 2.5 mL ethyl acetate, and 2.5 mL methanol were used at a distance of 9 cm. For the analysis of phenolic acids, 3 mL solution B (heptane: dichloromethane, 7: 3), 2 mL diisopropyl ether, 0.1 mL 85% formic acid, and 1 mL distilled water were used in the development step 1 and 2. However, from step 3 to step 7, 4 mL solution B (heptane: dichloromethane, 7:3), 1 mL diisopropyl ether, and 0.1 mL 85% formic acid were used for development. Protocatechuic, vanillic, and cafeic acids were identified in L. *album subsp. album* flowers in two forms: as free acids and as glycosides. The highest amount of caffeic acid was found in the extract after basic hydrolysis. HPLC was used to separate the phenolic acids using the RP-18 column and water-methanol-formic acid (75: 25: 0.5, *v*/*v*/*v*) as mobile phase with UV detection at 254 nm. Nine acids were detected in esterified forms, namely protocatechuic, vanillic, caffeic, syringic, gallic, gentisic, p-coumaric, and chlorogenic acids, and trace amounts of ferulic acid [56].

UHPLC-DAD-ESI-HRMS/MS combined with molecular networking-based dereplication and NMR were applied for the identification of flavonoid-3-O-glycosides in the ethyl acetate fraction of the methanolic extract from leaves of *Casearia arborea*, which is a fundamental source of cytotoxic highly oxidised clerodane diterpenes, besides phenolics, flavonoids and glycoside derivatives [58]. The scholars identified compounds not annotated in the GNPS platform (global natural products social molecular networking) by co-injection of standards in HPLC-DAD or via isolation and characterization of the metabolites using NMR spectroscopy. HPLC-DAD analysis was performed on the RP Zorbax Eclipse Plus column using water (0.1% acetic acid) with acetonitrile in gradient elution. Detection was done at λ = 352 nm. A molecular family of six flavonoid-3-O-glycosides in the molecular networking formed by the GNPS platform was annotated. They were avicularin, cacticin, isoquercitrin, quercitrin, rutin and a quercetin-3-O-pentoside cluster. The annotations with standard compounds by HPLC-DAD co-injection three flavonoid-3-O-pentosides and characterisation via 1D and 2D NMR was confirmed, except for identifying quercetin-3-O-robinobioside and kaempferol. Furthermore, three flavonoid-3-O-pentosides were isolated and characterized by 1D and 2D NMR spectroscopy. They were identified as reynoutrin, guaijaverin and avicularin [58].

The utility of off-line 2,2 -diphenyl-1-picrylhydrazyl (DPPH)-gas chromatography (GC)-mass spectrometry (MS) offline and the high-speed countercurrent chromatography (HSCCC) method for screening and identification of separation of antioxidants in essential oil of *Curcuma wenyujin* was performed by Y.H. Chen et C. Ling [62]. The essential oil was analyzed by means of the HP-5 5% phenyl methyl siloxane capillary column and GC chromatograph with a flame ionization detector (FID). The five compounds were described as antioxidants using

DPPH-GC and were next identified with the GC-MS method. Eucalyptol, camphor, δ-elemene, β-elemene, and curzerene were successfully isolated by the HSCCC method with the use of the following solvents: *n*-hexane-acetonitrile-ethanol (5:3:2, *v*/*v*) and *n*-hexane-acetonitrile-acetone (4:3:1, *v*/*v*). Finally the antioxidant property of theme have been confirmed by DPPH scavenging assay.

The HPLC-SPE-NMR technique was applied for the analysis of furanosesquiterpenoids from bark exudates of the tree *Commiphora swynnertonni* Burrt, which is used by ethnic groups in Tanzania and Kenya to treat various diseases in humans and to kill ectoparasites infesting livestock [63]. Separation was carried out using a silica-based Apollo C18 column and mobile phase consisting in water with 0.1% formic acid and acetonitrile in gradient elution. Detection was done at λ = 246 nm. Four known furanosesquiterpenoids, namely 6-oxodendrolasin, *(E)*-6-oxoisodendrolasin, *(Z)*-6-oxoisodendrolasin, crassifolone and one new one, namely 7,8-dihydroisodendrolasin, were obtained, and were stated to constitute a main part of the exudate [63].

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