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Identification in drug quality control and drug research

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ABSTRACT

Identification is an important step in the quality control of drugs and the research for new drugs. First, this review discusses the identification of bulk drugs and the active ingredients in formulations, based mainly on pharmacopoeial tests. The most important methods for this purpose are infrared (IR) and, to a lesser extent, ultraviolet (UV) spectroscopy, as well as retention matching with standards using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). The identification of impurities and degradants is based mainly on HPLC-UV, HPLC-mass spectrometry (HPLC-MS) and nuclear magnetic resonance (NMR) spectroscopies. The above methods are also used for identification purposes in drug research. The use of MS and NMR in the research for large-molecule drugs of biotechnological origin and natural products, mainly of plant origin, with special respect to traditional Chinese (and Indian) medicines is also discussed. The review concludes with the identification aspects of the fight against counterfeit drugs.

Contents

1. Introduction

Identification is the first step in the complex procedure of drug quality control and in the quality aspects of the search for new synthetic drugs and natural products. It is also an important tool in the fight against counterfeiting of drugs. It is sometimes difficult to find the borderline between identification and structure elucidation. The European Pharmacopoeia presents a good answer to this question: "*The tests given in the Identification section are not designed to give a full confirmation of the chemical structure or composition of the product; they are intended to give confirmation, with an acceptable degree of assurance that the article conforms to the description on the label*" [1].

Although, especially in the case of new drugs, drug research is the first step followed by production and quality control of the drug, in this review, the role of identification in drug quality control is dealt with first, because this issue is more important when drugs already exist, and, in drug research, structure elucidation is more important than identification.

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2. Identification in drug quality control

2.1. Identification of bulk drugs

This section is mainly based on the latest editions of the European Pharmacopoeia (Ph. Eur.) [1], which is the basis of the pharmacopoeias of the European Union (EU) countries, the US Pharmacopeia (USP) [2] and the Japanese Pharmacopoeia [3].

The monographs of bulk drugs in pharmacopoeias and in other main documents (e.g., Drug Master Files) usually begin with the identification test(s) because simple but reliable identification is of great importance in avoiding the danger of drugs from the manufacturing pharmaceutical company being confusing to the pharmacy where they are sold.

2.1.1. Classical color or precipitation reactions

A few decades ago, when spectroscopic and chromatographic methods were not yet developed enough for drug-identification purposes, usually test-tube methods based on color and precipitation reactions were used for this purpose. Some of these are still used in USP, as a possibility to be used in less developed places as test *B*, *C* or *D*, where *A* is a more up-to-date method. This is characteristic of the treatment of classical drug materials. For example, in the case of morphine sulfate, the following methods are still official in the latest edition of USP [2]: *"**B:** To 1 mg in a porcelain crucible or a small dish add 0.5 mL of sulfuric acid containing, in each mL, one drop of formaldehyde TS: an intense purple color is formed at once, and quickly changes to deep blue-violet (distinction from codeine, which gives at once an intense violet-blue color, and from hydromorphone, which gives at first a yellow to brown color, changing to pink and then to purplish red.) **C:** To a solution of 5 mg in 5 mL of sulfuric acid in a test tube add 1 drop of ferric chloride TS, mix, and heat in boiling water for 2 minutes: a blue color is produced, and when one drop of nitric acid is added, it changes to dark red-brown (codeine and ethyl morphine give the same color reactions, but hydromorphone and papaverine do not produce this color change). **D:** A solution (1 in 50) responds to the test of Sulfate <191>."].*

More or less the same applies to the Ph. Eur. In this case sets of First and Second Identification are included in many monographs: *"Certain monographs have subdivisions entitled 'First identification' and 'Second identification'. The test or tests that constitute the 'First identification' may be used in all circumstances. The test or tests that constitute the 'Second identification' may be used in pharmacies provided it can be demonstrated that the substance or preparation is fully traceable to a batch certified to comply with all the other requirements of the monograph. Certain monographs give two or more sets of tests for the purpose of the first identification, which are equivalent and may be used independently".*

In the case of morphine sulfate, the above-mentioned sulfate test is included in the First and the formaldehyde-sulfuric acid test in the Second Identification set.

The sulfate test (precipitation with barium chloride) appears in both present official pharmacopoeias. While the importance of the color and precipitation reactions has greatly decreased and is replaced by modern spectroscopic and chromatographic methods to be discussed in the following sections, some of these tests are rather widely used, mainly in the identification of salts based on their counter-ion.

In addition to the above-mentioned sulfate test the identification of chlorides as silver-chloride precipitate is still widely used.

In addition to these, the Ph. Eur. contains identification tests based on color or precipitation reactions to the following anions, cations and some other items: acetate, acetyl group, alkaloids, aluminum, amines (primary aromatic), ammonium, antimony, arsenic, barbiturates, benzoate, bismuth, bromide, calcium, carbonate and bicarbonate, citrate, esters, iodide, iron, lactate, lead, magnesium, mercury, nitrate, phosphate, potassium, salicylate, silicate, silver, sodium, tartrate, xanthines and zinc. The USP contains tests for the same items with the addition of some others, such as barium, borate, chlorate, cobalt, copper, hypophosphite, lithium, manganese, nitrite, oxalate, permanganate, peroxide, sulfite, thiocyanate and thiosulfate.

2.1.2. Spectroscopic methods

Infrared spectroscopy (IR) is undoubtedly the most widely-used identification test for bulk pharmaceuticals in all modern pharmacopoeias and other documents, since this method is rapid and, because IR spectra are very rich in bands of different intensities as a function of the functional groups and their structural environment, it is highly characteristic. In the majority of cases, the potassium-bromide disc method is used: the spectrum of the investigated material should be identical with that of the Reference Standard, obtainable from the related Pharmacopoeia Commissions. It is worth mentioning that, in the case of the Japanese Pharmacopoeia [3], it is not important to scan the spectrum of the Reference Standard, since this pharmacopoeia contains 560 IR spectra for comparison purposes.

Polymorphism, frequently occurring among pharmaceuticals, causes a problem that is not too serious when using IR spectroscopy for identification purposes, since there are minor differences between the IR spectra of the polymorphic modifications taken in the solid state. In the majority of cases, the bioavailability of the pharmaceuticals does not greatly depend on the polymorphic modification. For this reason, in a case of observing minor difference between the spectra of the tested sample and of the Reference Standard, all pharmacopoeias usually prescribe repetition: the two samples should be dissolved in the same volatile solvent and, after evaporation to dryness, the spectra should be scanned again. In the case of equivalence of the spectra, the test is successful.

Although less generally used than IR, ***ultraviolet spectroscopy (UV)*** is also an important method for the identification of drugs in pharmacopoeias. No doubt that the UV spectrum is less characteristic of the investigated compound than the IR spectrum. To increase the reliability of this test, it is sometimes prescribed that the absorbance at the given maximum value should be within ±3% of that of the Reference Standard and, in some cases, the absorbance ratio at two characteristic wavelengths is also limited. An example for the latter is atenolol in the Ph. Eur. [1], where the absorbance ratio at the two characteristic maxima of the phenol-ether moiety (A_{275}/A_{282}) should be within the limits 1.15–1.20.

Nuclear magnetic resonance spectroscopy (NMR) is only used in a few cases in pharmacopoeias for identification purposes, especially for large molecules. Examples are heparin, goserelin and buserelin in the Ph. Eur., and heparin, enoxaparin and oxytocin in the USP.

The determination (with limits) of the ***optical rotation***, mainly at the sodium D-line at 589,3 nm is part of the identification of many optically-active compounds in Ph. Eur., while this is one of the other tests in the USP and the Japanese Pharmacopoeia outside the Identification tests. (The same applies to the non-spectroscopic method, determination of the ***melting point***, also with limits.).

2.1.3. Chromatographic methods

In addition to the progress in spectroscopy, the developments within chromatography [4] have also made great changes in the identification of drugs.

High-performance liquid chromatography (HPLC) is the most generally used method for the assay of bulk drug materials in the USP with lesser but still important contribution in Ph. Eur. and the Japanese Pharmacopoeia. Although the value of the results obtained by HPLC is questionable due to precision problems when these

methods are used for assaying the bulk drug materials [5–8], HPLC can be successfully used for identification purposes. The retention time of the peak of the sample solution should be identical with that of the standard solution. If the assay method is not a HPLC test, but the test for limiting the organic impurities is based on it, HPLC is often prescribed (especially in Ph. Eur.) for the identification of the drug based on the same principle. A great advantage of an identification method of this kind is that no separate work is necessary: the comparison can be made in the course of the assay and tests for organic impurities.

The test for the determination of organic impurities in pharmacopoeias is often based on ***thin-layer chromatography (TLC)***. This test can also be used for identification purposes: the identity of the drug materials is proved by the identity of the R_F values of the main spots in the chromatograms of the sample and standard solutions. It is interesting (and difficult to understand) that separate TLC identification tests are described in many cases when the monograph contains suitable HPLC or TLC tests that could be used without extra work for identification purposes. Although the importance of ***gas chromatography (GC)*** is not comparable with that of HPLC and TLC in the identification of bulk drug materials, this method can be found in the pharmacopoeias for the identification of some volatile and less polar compounds.

2.1.4. Electromigration methods

The importance of these methods in the identification of bulk drugs is not comparable with the chromatographic methods but there are some cases of their use in the pharmacopoeias, mainly for biotechnology-derived macromolecules. For example, ***agarose gel electrophoresis*** is used in Ph. Eur. for the identification of chondroitin sulfate while ***isoelectric focusing*** is used for the identification of molgramostim, and ***capillary electrophoresis (CE)*** is used for proteins, such as somatropin and erythropoietin, in the same pharmacopoeia [1].

2.2. Identification of the active ingredients of pharmaceutical products

The methods used for the identification of the active ingredients in pharmaceutical products are usually identical to those of bulk drug materials. The information available is less than in the former case: Ph. Eur. does not contain monographs for pharmaceutical products and companies typically do not publish their analytical protocols. On the basis of USP and the Japanese Pharmacopoeia, the main methods for the identification are IR and, to a lesser extent, UV spectroscopy and classical color reactions, as well as HPLC and TLC retention matching with standard materials. Depending on the formulation, it may sometimes be necessary to extract or to evaporate to dryness the formulation prior to using these methods. It may be necessary to dissolve the tablet formulation and precipitate the active ingredient prior to the use of IR for identification. The sample preparation is sometimes quite laborious. For example. in the case of amantadine hydrochloride capsules, in the USP, the content of the capsule should be dissolved in 0.1 N HCl, filtered, made alkaline with 5 N NaOH and extracted with methylene chloride. In this (and many similar cases), the IR spectrum is taken in solution state. In the case of aminocaproic acid oral solution, the active ingredient is separated by ion-exchange chromatography before IR spectroscopy in the solid state.

A great advantage of the rapid identification of the active ingredients, especially in solid dosage forms, by near-infrared (NIR) spectroscopic analysis is that this is a direct method: no extraction is necessary, enabling, e.g., the investigation of tablets in blisters without removing the tablets [9–11].

2.3. Identification of impurities in drugs

Due to the above problems with the ***assay*** methods of drug materials [5–8], ***impurity profiling*** is certainly the most important part of the quality-control documents of bulk drugs. The threshold above which the impurities should be identified is prescribed in the document presented by ICH (International Conference on Harmonization), dealing with the harmonization of regulatory expectations in the USA, EU and Japan [12]. In the case of drugs administered at a level of ≤2g/day, this threshold is 0.10% or 1.0 mg per day intake (whichever is lower), while, in the case of >2g/day, it is 0.05%. Identification here means definitive structure elucidation.

The importance of this field is shown by several books [13–15], book sections, special issues in journals [16,17] and reviews [18,19] devoted to it. The number of references are 257 [18] and 266 [19]; and, the total number of papers dealing with this field is >1000.

Fig. 1 shows the scheme [20] of the way in which impurities are generally identified. As is seen, after detection of the impurity by HPLC or TLC (or another chromatographic or related method), the identification is attempted by retention matching with the aid of available potential impurities. If this is unsuccessful, it is advisable to draw as much conclusion as possible regarding structure from the UV spectra obtainable on-line with the aid of HPLC diode-array UV detection [21,22].

The main methods for the identification/structure elucidation of the unidentified impurities are mass spectrometry (MS) and NMR spectroscopy. In the earlier (but still widely used) ***off-line*** method, the impurities are isolated by (semi)-preparative HPLC before obtaining their MS and NMR spectra. An example is shown in Fig. 2. An impurity found in lovastatin using the HPLC purity test of Ph. Eur. [1] was separated by preparative HPLC followed by structure elucidation by MS and NMR. The structure found characterizes a by-product of the biosynthesis of lovastatin by fermentation [23].

Among the ***on-line*** studies, there are many where HPLC-(UV)-MS/(MS) provides sufficient information for the identification and the structure elucidation of the impurities {e.g., the investigation of vertilmicin by HPLC coupled to MS with electrospray ionization enabling the identification of 18 impurities (among them 11 new ones) [24]}. For a review of this field, see [25].

In the majority of cases, ***semi-on-line*** solutions of the impurity identification problems are described: in addition to on-line HPLC-(UV)-MS/(MS) investigation some impurities are isolated by semi-preparative HPLC for NMR investigation. A typical example is the identification of impurities in bis-indol alkaloids vinblastine and vincristine [26]. Due to the high cost of the necessary instruments and some other problems [20,27], the fully ***on-line*** approach [HPLC-(UV)-MS/(MS) and LC-NMR] is used relatively infrequently {e.g., the identification of an N-cinnamyl impurity in icofungipen [28]}.

As shown in Fig. 1, the exact structure elucidation of the impurities is usually completed by the synthesis of the impurities and retention and spectral matching with those found in the drug material or drug product [20].

Genotoxic (or potentially genotoxic) impurities play a special role within the impurity profiling of drugs. Since these impurities are toxic at much lower concentrations than average impurities, the requirements are much stricter. For example, the “threshold of toxicological concern” of as low as 1.5 μg/day intake of such impurities is considered to be associated with health risk [29,30]. In the case of aflatoxin-like, N-nitroso and alkyl-azoxy compounds, the threshold values can be even lower [31].

2.4. Identification of degradants in drugs and drug products

Since degradants can be considered a subset of impurities that can be present in drugs or drug formulations, most aspects of drug-impurity profiling, including their identification, described in the

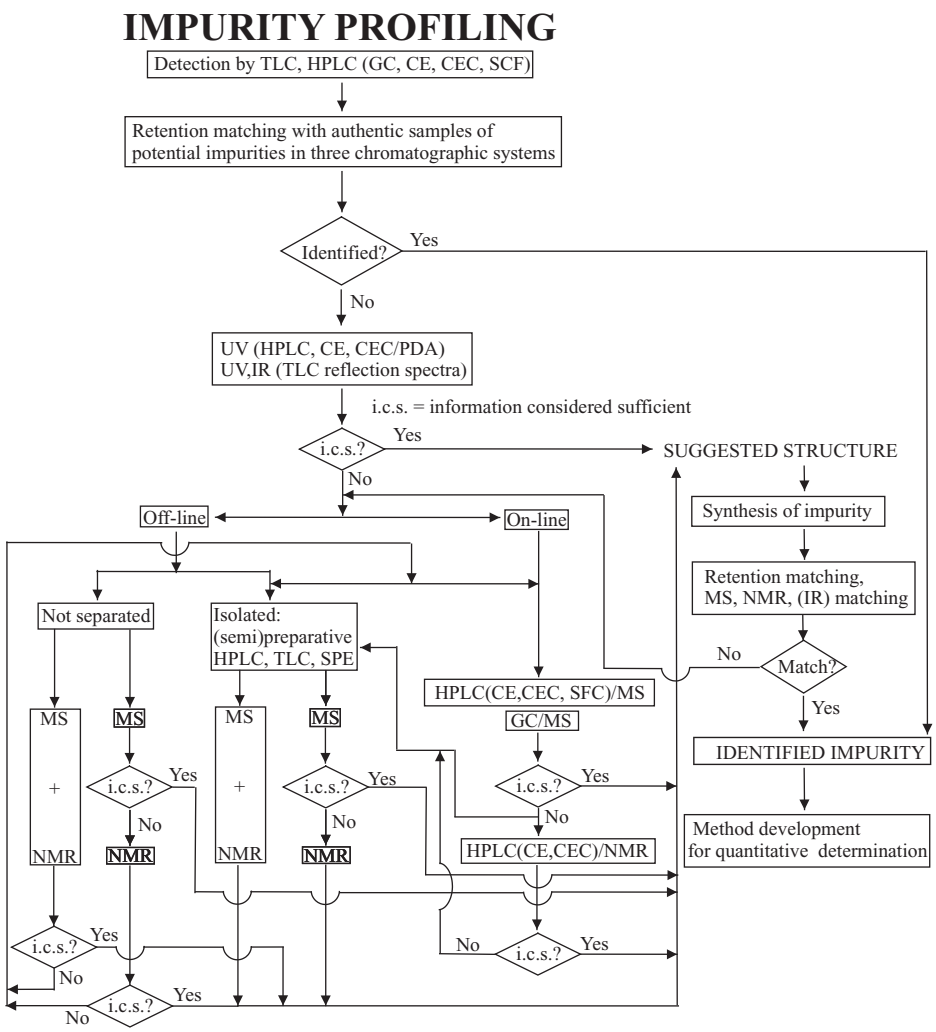


Fig. 1. A general scheme for the profiling of related organic impurities in drugs involving the use of various spectroscopic and chromatographic techniques. {From [20] with permission}.

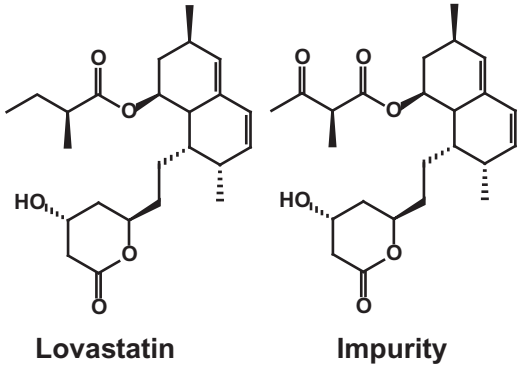


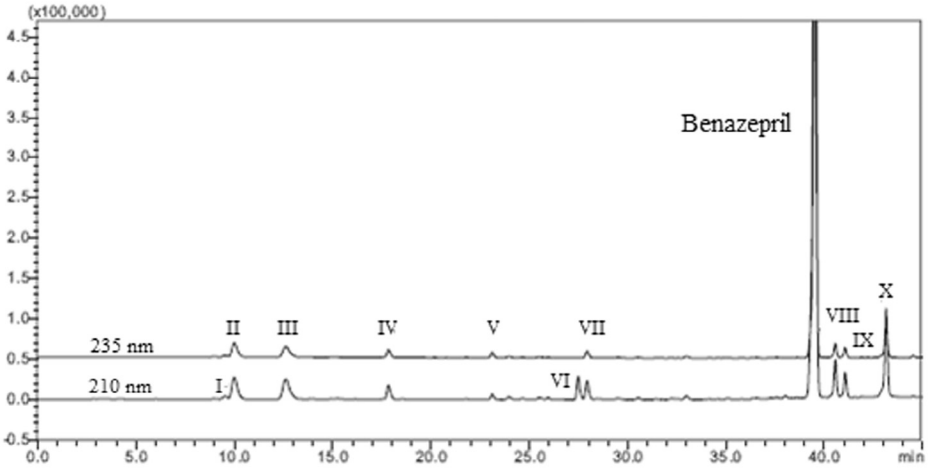
Fig. 2. Structures of lovastatin and its impurity [23].

previous section also relate to degradants. A Special Issue of this journal was recently devoted to this aspect [32]. Of the several books, reviews and papers referenced in this Special Issue, a recently published book [33] and two reviews [25,34] are mentioned here.

The book [33] and three other reviews [35–37] deal with ***forced (or stress) degradation*** studies. The identification and the structure elucidation of (potential) degradants formed in the course of these studies is an important step in the development of a new drug or a dosage form in order to see the degradation pathway of the drug substance under more severe conditions than the normal storage conditions. This method enables prediction of real impurities formed in the course of storing the drug under normal conditions. For stress-degradation studies, drug authorities prescribe storage of the drug substance or drug product at various high temperatures and various relative humidities, checking the susceptibility of the drug substance to hydrolysis by treating solutions or suspensions at a wide range of pH, and susceptibility to oxidation in the presence of an oxidizing agent (preferably hydrogen peroxide) also at elevated temperatures. Stress testing should also include testing of the sensitivity of the drug substance to light. In addition to the above points, it is important to note that, with this information, the stability-indicating nature of the assay method can be checked [38].

Of the innumerable publications in the literature, two are shown here as typical examples, as follows.

- Complex use of the above techniques (preparative and analytical HPLC, various LC-MS techniques, and LC-NMR) is for the elucidation of the stress-degradation profile of benazepril [39]. Fig. 3 shows the HPLC chromatogram with the degradation products found under different stress conditions, while Fig. 4 shows the degradation pathway derived from these studies.
- The other example is the structure elucidation of minor oxidative degradants of the classical drug material, estradiol, by the



Degradation product peaks	I	II	III	IV	V	VI	VII	VIII	IX	X
Stress conditions	PA, PN	PA, PN	PA, PN	PA, PN	PA, PN	PA, PN	A, B, N	PA, PN	PA, PN	PA, PN

Fig. 3. HPLC chromatogram showing different degradation products (I–X) of benazepril at two wavelengths (210 nm and 235 nm). Key: A: acid; B: base; N: neutral; PA: photoacid; PN: photoneutral. {From [39] with permission}.

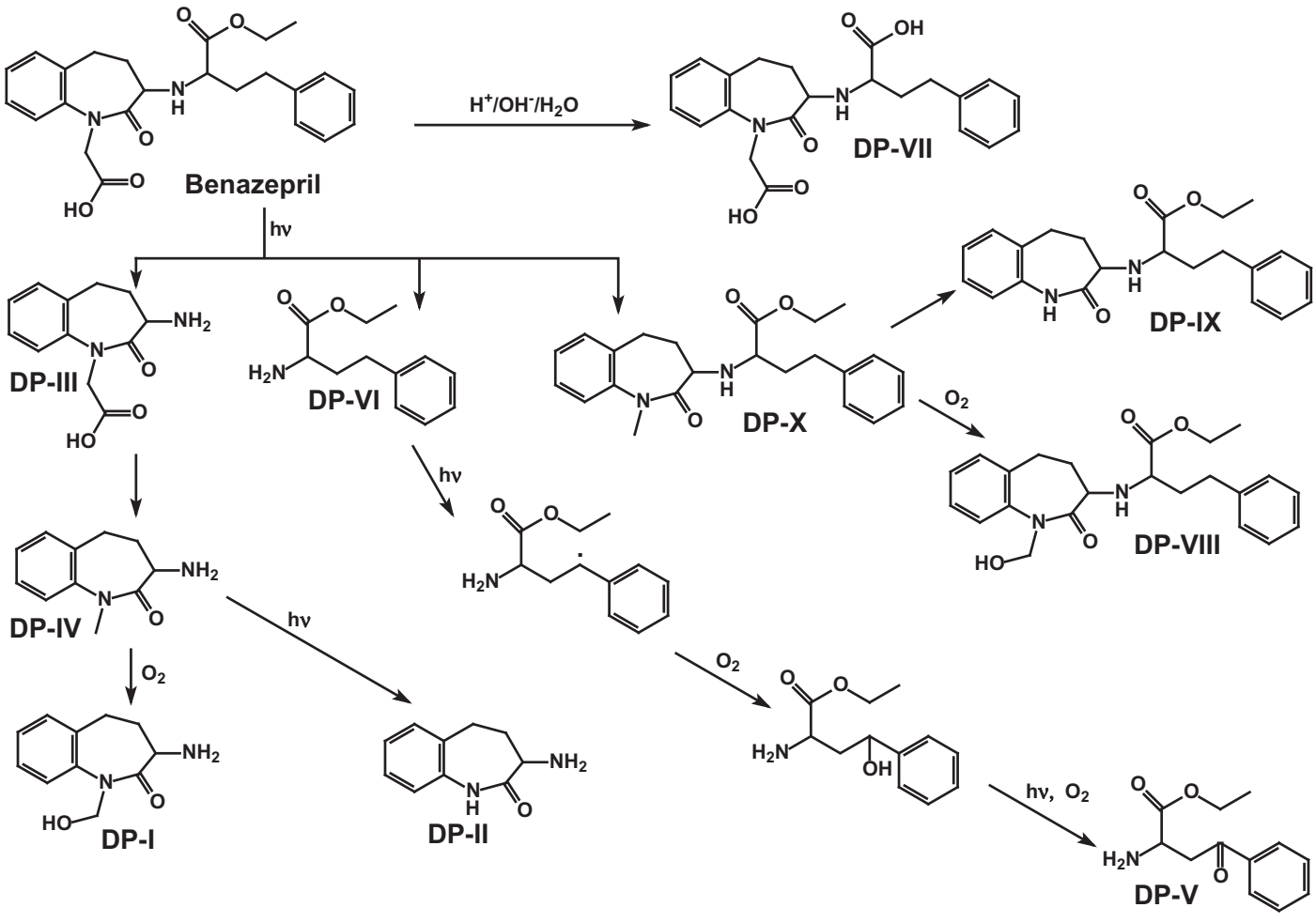


Fig. 4. Degradation pathway of benazepril (DP = Degradation product) [39].

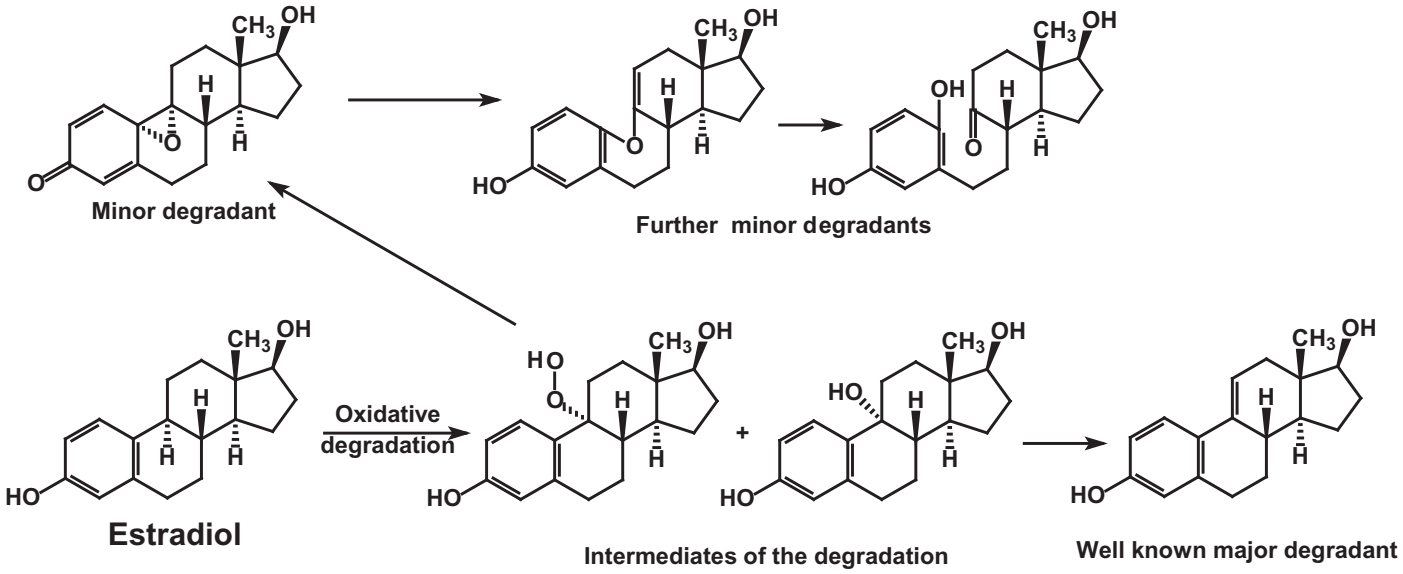


Fig. 5. Degradation pathways of estradiol [21,40,41].

complex use of the above techniques [40]. Fig. 5 shows the mechanism of the oxidative degradation. The structure of the major degradant was determined earlier using HPLC-UV and MS methods [21,41].

3. Identification in drug research

3.1. Synthetic products

Despite the increase in the importance of macromolecules in drug therapy, the majority of the drugs used at present are small molecules. The structure elucidation and the identification of intermediates and end products are described in the documents submitted to drug authorities. What is described in the pharmacopoeias and in Section 2.1 of this article is also valid for these compounds.

The analytical aspects of combinatorial and parallel syntheses of drugs [42] merit special mention. These are important methods in drug research and the characterization of the "libraries" obtained, containing thousands of compounds, including identification of synthesized compounds, is a very special field of drug analysis [43]. The most widely-used methods for this purpose are MS [43–45] and NMR spectroscopy [43,46], coupled to HPLC.

3.2. Biotechnological products

As already mentioned, the importance of macromolecules, mainly proteins (monoclonal antibodies and others), of biotechnological origin is increasing within drug therapy. In their identification, the classical spectroscopic methods generally used for classical small molecule drugs (IR and UV) are replaced by MS [47,48] and NMR [49].

When briefly mentioning biotechnological protein drugs, it is also important to mention ***biosimilars*** produced by competitors of the originators using more or less different biotechnological procedures for their production. The potency of biosimilars against various diseases is very similar to that of the reference product with also highly similar purity and safety profile [50]. The use of various spectroscopic (mainly MS after enzymatic splitting or investigation of the intact molecules by tandem MS), chromatographic, electrophoretic and other analytical methods in the identification of the main, bioactive components and their impurities was summarized in a recently published review [51].

3.3. Natural products

Although, as described in sub-sections 3.1 and 3.2, in our age, the main targets of drug research are synthetic products (small molecules and biotechnologically-produced proteins), compounds of natural (mainly of plant) origin also play a fairly important role. Hundreds of publications deal with the separation, identification, structure elucidation and quantitative analysis of the active ingredients of mainly Chinese and Indian traditional drugs. Since, according to the theory of Traditional Chinese Medicine (TCM), the therapeutic effect of these drugs can be attributed to the simultaneous presence of several active ingredients, their identification is generally part of a complex procedure. One of the possibilities is fingerprinting based mainly on chromatographic methods used for herbs with relatively well-known constituents [52–54].

For the identification of constituents of less-known herbs and their combinations, on-line coupling of chromatographic methods (mainly HPLC) with MS and sometimes also NMR after separation by preparative HPLC are used [55–57]. An example of the first type is a paper in which 81 constituents (organic acids, amino acids, oligosaccharides, alkaloids, nucleosides, phenylpropanoids, polyacetylenes, flavonoids, isoflavonoids and saponins) of the TCM Shenqi Fuzheng Injection were identified by means of UHPLC coupled with electrospray-ionization quadrupole time-of flight mass spectrometry (UHPLC-ESI-QTOF-MS) [58]. An example where NMR also needed to be used for the identification of some constituents was the analysis of the TCM Ju-Zhi-Jiang-Tang. HPLC and UHPLC coupled with QTOF-MS and ion-trap MS (IT-MS) were able to identify 151 compounds (e.g., flavonoids, terpenoids, phenylpropanoids, organic acids, alkaloids, cyclopeptides, and oligosaccharides), of which 18 were novel. Some of their structures could only be tentatively identified by MS, but were confirmed by NMR after separation by semi-preparative HPLC [59].

4. Counterfeit products

Detecting and proving the adulteration of drugs are among the most serious problems for pharmaceutical analysis [60]. Analytical chemists play a very important role in the fight against drug adulteration: identification of the constituents of the adulterated drugs is one of the most important tasks.

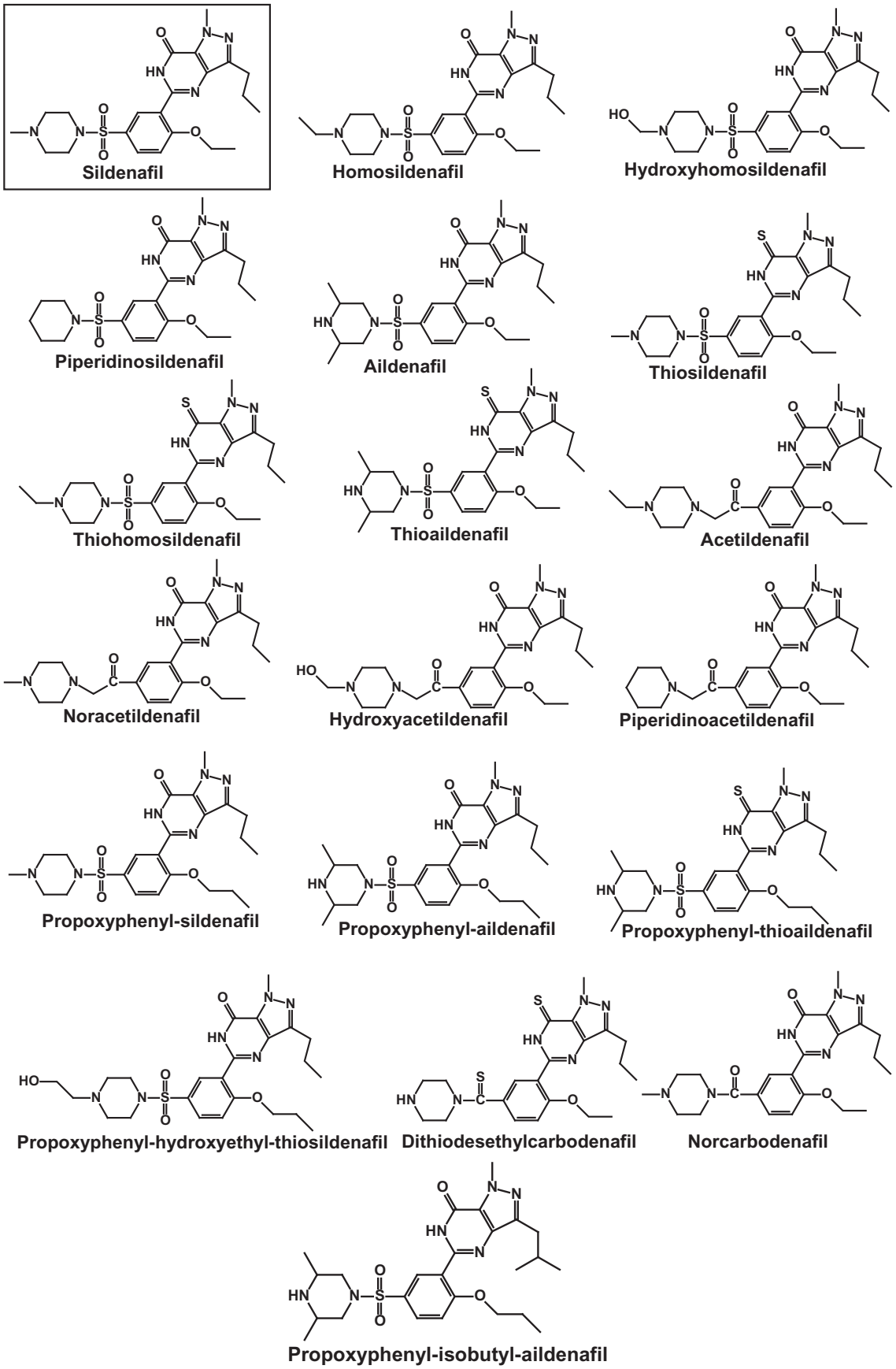


Fig. 6. Structures of sildenafil and some of its illegally used analogues [62–67].

Due their great popularity, drugs against erectile dysfunction (e.g., sildenafil citrate: Viagra and tadalafil: Cialis) are often falsified and illegally sold using bulk material not prepared in an environment with good manufacturing practices (GMPs). The detection of this type of adulteration is possible on the basis of impurity profiling (i.e., identifying and quantifying the impurities) that are always present in higher numbers and greater quantities in illegal products than in bulk drugs and drug formulations manufactured under GMP conditions [61].

Even more dangerous for human health is the adulteration of herbal drugs and herbal dietary supplements, advertised as "all natural", by the above synthetic drugs (e.g., against erectile dysfunction, anti-obesity drugs, psychoactive drugs, anabolic steroids), but not indicated in their ingredients list. In the worst, but very frequent, case, herbal drugs are adulterated by adding to them the above drugs with slightly modified structures which are more difficult to detect than the structures of the original drugs. There are more than 50 of these analogues of erectile dysfunction drugs (sildenafil, tadalafil, vardenafil) with numerous and unpredictable adverse effects [62].

It is not the aim of this article to make reference to all papers dealing with adulteration (of the order of 100). A characteristic example is a review paper summarizing the literature before 2009 [63], in which 11 sildenafil analogues were said to have been illegally administered in herbal drugs and herbal dietary supplements (i.e., homosildenafil, hydroxyhomosildenafil, piperidinosildenafil, aildenafil, thiosildenafil, thiohomosildenafil, thioaildenafil, acetildenafil, noracetildenafil, hydroxyacetildenafil, piperidinoacetildenafil). Since the publication of this review [63], further illegal derivatives were identified in herbal drugs [i.e., propoxyphenyl-sildenafil [64], propoxyphenyl-aildenafil, propoxyphenyl-thioaildenafil, propoxyphenyl-hydroxyethyl-thiosildenafil [65], dithiodesethyl-carbodenafil, norcarbodenafil [66] and propoxyphenyl-isobutyl-aildenafil [67]}. Fig. 6 shows some of the structures identified.

The identification of these derivatives is done as described in sub-section 2.3, but the use of FT-IR [68,69], NIR [69,70], Raman [69,71–73], NMR [74] spectroscopies and X-ray diffraction analysis [75] has also been described.

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