

Review Article

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Review of characteristics and analytical methods for determination of indomethacin

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Abstract: Nonsteroidal anti-inflammatory drugs (NSAIDs) are the first choice of treatment for rheumatic disorders and other degenerative inflammatory diseases. One of them, indomethacin (INDO), is highlighted in this study. With its analgesic, antipyretic, and anti-inflammatory properties, it is one of the most powerful drugs used in various clinical trials and therapies related to the mechanism of blocking prostaglandin synthesis, thus reducing and eliminating many inflammatory conditions in patients. To ensure the efficacy and safety of this drug in pharmaceutical and clinical use, precise product quality control is required. Such control is performed with routine pharmaceutical analysis using various chemical methods by which INDO is identified as a separate active ingredient in the multicomponent system of a complete pharmaceutical form. In addition, the determination of INDO is important in clinical practice, where its concentration is determined in different biological samples, ensuring better monitoring of a particular therapy. The most commonly used methods for the determination of INDO are high-performance liquid chromatography (37% of developed methods), voltammetry (16% of developed methods), and UV spectroscopy (11% of developed methods). However, each of these methods must provide precise validation parameters. A combination of analytical methods can lead to more precise results and safer application in practice.

Keywords: nonsteroidal anti-inflammatory drugs, indomethacin, analytical methods

1 Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a group of drugs with a similar mode of action but often without structural similarity [1]. They have analgesic, antipyretic, and anti-inflammatory effects and are the first choice of treatment for rheumatic disorders and other degenerative inflammatory diseases. The term NSAID was used for the first time in 1949 [2]. Classification is most commonly based on chemical structure and selectivity: salicylates, nonacetylated salicylates, propionic acids, acetic acids, enolic acids, anthranilic acids, naphthylalanine, and selective cyclooxygenase-2 inhibitors [3]. Their mechanism was described for the first time in 1971 [4–6], when it was shown that this type of drug inhibits the biosynthesis of prostaglandins by preventing binding of the substrate, arachidonic acid, to the active site of the enzyme cyclooxygenase (COX) [7].

Indomethacin (INDO) is an NSAID that belongs to a group of acetic acid derivatives. It possesses anti-inflammatory activity and chemopreventive properties and also inhibits the enzyme COX. As an NSAID, INDO may inhibit the expression of multidrug-resistant protein type 1, which leads to increased efficacies of some antineoplastic agents in treating multidrug resistant tumors [8]. Also, INDO has gained great attention from different research groups as a potential treatment or adjunct therapy for severe acute respiratory syndrome coronavirus-2/coronavirus disease 2019 (SARS-CoV-2/COVID-19) [9–11]. In April 2020, a phase II clinical study [12] was started with the aim of elucidating the efficacy and safety of oral INDO in SARS-CoV-2-positive patients with mild COVID-19 symptoms. Zeng et al. [9] reported INDO as a potential repurposable drug candidate for COVID-19. Generally, drug repurposing is a strategy that enables the identification of new uses for approved or investigational drugs [13]. The authors proposed a knowledge-graph-based deep-learning methodology for drug repurposing in COVID-19 as an integrated artificial intelligence methodology. Using the proposed methodology, the authors identified 41 drugs, including INDO. The therapeutic

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associations of these drugs with COVID-19 were validated by transcriptomic and proteomics data in SARS-CoV-2-infected human cells and data from ongoing clinical trials. Although the new methodology represents a powerful tool to prioritize existing drugs for further investigation, all predicted drugs must be tested in clinical trials before using for COVID-19 treatment.

To safely use NSAIDs in the pharmaceutical industry and clinical therapies, it is necessary to develop accurate and reliable analytical methods for their determination in different types of samples. An important purpose of these methods is to provide qualitative and quantitative information about the drug of interest to determine its purity, pharmacokinetics, and pharmacodynamics. As well known, drugs are subject to biotransformation. The resulting metabolites that can be more toxic than the initial drug can be found in the environment; therefore, environmental monitoring of drugs is also necessary.

Developed methods should be simple, inexpensive, and environmentally friendly. A large number of samples should be able to analyze in a short time, and sample preparation, as one of the most important steps of the analysis, should be simple and fast. The emphasis should also be on selectivity due to the complexity of the samples and the possible influence of the interferences.

This study presents a review of the characteristics and properties of INDO and, for the first time, highlights the analytical methods described in the literature for

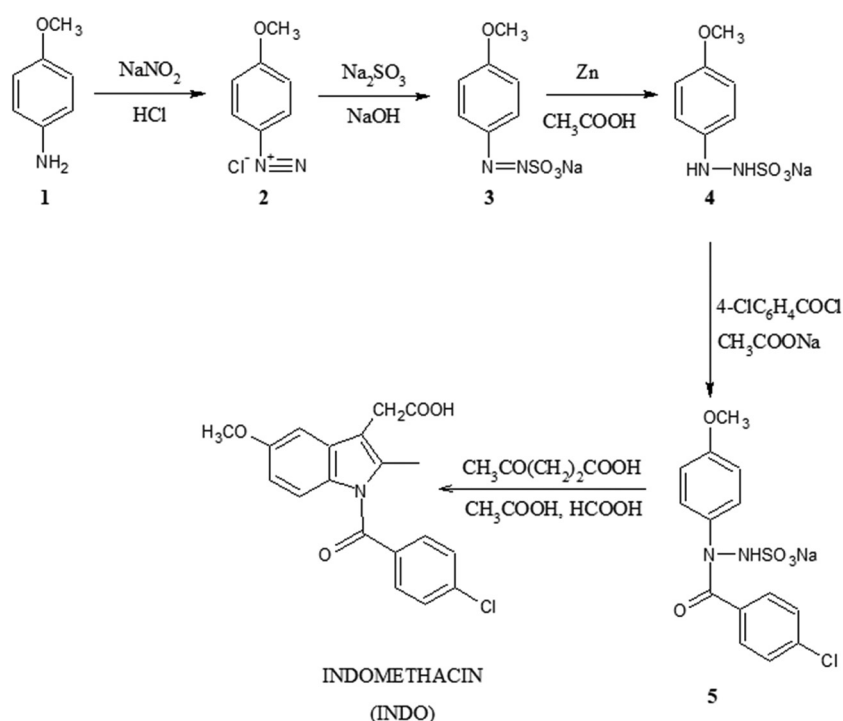
INDO determination between 2000 and 2020. Methods developed between 1965 and 1999 were also studied to present the overall statistics of the developed methods for INDO determination. To the best of our knowledge, there are no other review articles focused on analytical methods for the determination of INDO. The research for this review was based on the following databases: Scopus, Web of Science, and Pub Med, using INDO determination as the main keyword.

This review gives preliminary data about INDO characteristics and its determination, so it could be helpful as a first step in the development of new and improved analytical methods for INDO determination. Also, it could be very useful for researchers to realize the key concepts in INDO determination.

2 INDO

2.1 Structural form

INDO is an indole-acetic acid derivative present as [1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid, which was patented in 1961 and approved for medical use in 1963 [14,15]. Its structure is shown in Scheme 1.



Scheme 1: Synthetic procedure for preparation of INDO [16].

Magedov and coworkers in 2005 proposed a new procedure for INDO synthesis. As starting material, 4-methoxyaniline (**1**) was used, which was diazotized with the formation of 4-methoxybenzenediazonium chloride (**2**), which was converted into sodium 4-methoxybenzenediazosulfonate (**3**). In the next step of the reaction pathway, compound **3** was reduced with zinc dust, which resulted in the formation of sodium 2-(4-methoxyphenyl)-1-hydrazosulfonate (**4**). Sulfonate **4** reacted with 4-chlorobenzoyl chloride, which led to the formation of sodium 2-(4-chlorobenzoyl)-2-(4-methoxyphenyl)-1-hydrazosulfonate (**5**). For the last step, which included indolization of hydrazine **5** into INDO, it was necessary to select an acid catalyst that should have two reaction roles. The first role was to remove sulfonate protection from the nitrogen atom, forming a hydrazone on reaction with levulinic acid, and the second role was catalysis of the Fisher reaction without deacylating the 4-chlorobenzoyl group that was achieved by using formic acid as a catalyst (Scheme 1) [16].

2.2 Physicochemical properties

INDO is a white or yellow crystalline powder, which is almost insoluble in water and sparingly soluble in alcohol. It shows polymorphism and can exist as several crystalline forms. α -forms and γ -forms of INDO (melting range 152–154°C and 160–161°C) can be obtained from an array of solvents under various conditions [17]. γ -Form is stable, whereas α -form is metastable, INDO exists as a glassy amorphous form. Since they have different melting points, one way to identify the crystalline form of interest is to measure melting point. The more convenient way is single crystal X-ray diffraction and X-ray power diffraction. Also, one way to identify the different crystalline forms is to react with ammonia gas. Metastable α -form reacts with ammonia gas, but produces the corresponding microcrystalline ammonium salt, while the stable, γ -form is inert to ammonia gas. The glassy amorphous form reacts with ammonia gas to yield a corresponding amorphous ammonium salt [17].

INDO has a molecular formula $C_{19}H_{16}ClNO_4$, with a molecular weight of $357.8 \text{ g}\cdot\text{mol}^{-1}$. Its melting range is 158–162°C, depending on the form, its $\log P$ is 4.27, and its pK_a is 4.50 [18–21].

2.3 Structural modifications

In 2004, Rimmel et al. [22] reported that the methyl (CH_3) group at the 2-position of the indole ring in INDO sits in

hydrophobic pocket of COX enzyme. The replacement of the CH_3 group of the indole ring in INDO with an H atom resulted in a decrease in its anti-inflammatory activity [23].

In 2013, a new fluorine INDO analog (compound **6**, Figure 1a), bearing a trifluoromethyl (CF_3) group at the 2-position was prepared [24]. Replacement of the CH_3 group of INDO with CF_3 decreased its inhibitory activity toward COX-1, whereas its COX-2 activity was unchanged. Based on the results, further investigations were focused on examination of impact of introducing other fluorine-containing substituents at 2-position of the indole ring, such as a 3,3,3-trifluoroprop-1-enyl group. It was assumed that introducing the 3,3,3-trifluoroprop-1-enyl group would enable hydrophobic interaction with the COX enzyme by increasing the surface area available to interact with its hydrophobic pocket. Also, the rigid unsaturated carbon framework of 3,3,3-trifluoroprop-1-enyl group would result in increased thermodynamic stability [25]. Fluorinated analog of INDO bearing a 3,3,3-trifluoroprop-1-enyl group at its 2-position (compound **7**, Figure 1b) was synthesized for purpose of evaluation its inhibitory activity toward the COX-1 and COX-2 enzymes *in vitro*. The results revealed that this fluorinated analog exhibited greater inhibitor activity and selectivity toward COX-2 than toward INDO. To the best of our knowledge, compounds **6** and **7** are not commercialized. Further investigations are focused on a deeper understanding of the differences between the COX-1 and COX-2 enzymes in terms of their pharmacological inhibition.

In the introduction part is mentioned potential anti-SARS-CoV-2 activity of INDO. The mechanism of antiviral action of INDO is unknown, but different hypotheses have been proposed. Amici et al. reported that INDO does not affect virus infectivity, binding or entry into host cells, but acts at an early stage of the coronavirus replication cycle, causing global repression of viral protein synthesis via ds-RNA-dependent protein kinase R-mediated pathway [26].

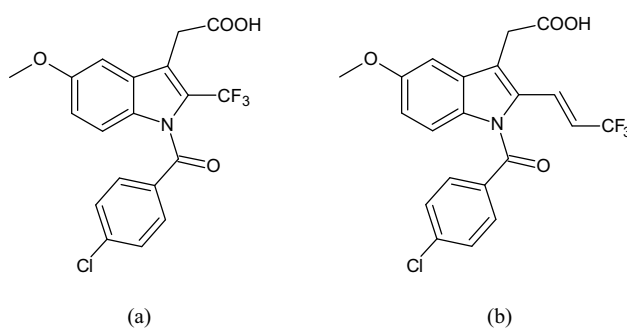


Figure 1: Chemical structure of compounds: (a) **6** and (b) **7**.

A recently published study on the SARS-CoV-2 interactome with human host proteins suggested that the anti-SARS-CoV-2 activity of INDO is related to its ability to inhibit prostaglandin E synthase type 2 (PGES-2) [27]. Results reported by Gordon and coworkers indicated that COVID-19 patients treated with INDO were less likely to require hospitalization than those treated with other anti-inflammatory drugs that do not target PGES-2 [28].

Anti-SARS-CoV-2 potency of INDO is limited in infected cells [29,30]. In the last few years, PROteolysis Targeting Chimeras (PROTACs) have emerged as a novel therapeutic modality in drug discovery. PROTACs are hetero-bifunctional molecules composed of a ligand for a protein of interest, a ligand binding to E3 ubiquitin ligase, and a linker that binds these two ligands [31]. Desantis and coworkers [30] decided to utilize INDO for designing PROTAC derivatives with the aim of investigation of this technology as a valid approach, also in the search for anticoronavirus agents. INDO inhibited the replication of SARS-CoV-2 with an EC_{50} (half maximal effective concentration) = $94.9 \mu\text{M}$. Despite INDO showing only a weak antiviral activity ($EC_{50} = 94.4 \mu\text{M}$), INDO-based PROTACs **8** (bearing a 6-methylene units linker) and **9** (bearing a piperazine-based linker) demonstrated an improved ability in inhibiting viral replication. Compounds **8** and **9** (Figure 2) resulted in the most potent compounds showing EC_{50} values of 18.1 and $21.5 \mu\text{M}$, respectively.

2.4 Mechanism of action

COX is an enzyme that catalyzes the first two steps of prostaglandin biosynthesis [32]. Prostaglandins are autacoids derived from arachidonic acid that are involved in

the maintenance of homeostasis and have a key role in the generation of an inflammatory response: fever, inflammation, promotion of pain, etc. [33]. The activity of INDO and other NSAIDs is based on the inhibition of prostaglandin synthesis. The key role of INDO in its mechanism of action includes blocking the synthesis of prostaglandins by influencing the activity of COX enzyme. COX has two isoforms: constitutive COX-1 and induced COX-2. The first one participates in the conversion of arachidonic acid to prostaglandin E2 and I2 and thromboxane A2. Based on that, COX-1 is characterized by cytoprotective activity toward the digestive system. Also, it has a positive effect on blood flow in the kidneys and regulates the activity of platelets. COX-2 is important for prostaglandin formation during pathophysiologic states, such as inflammation and tumorigenesis [32]. Besides that COX-2 has a role in forming prostaglandins that induce an increase in vascular permeability, edema, and pain (Scheme 2) [34]. The main mechanism of action of INDO is the reversible inhibition of COX-1 and COX-2 [1]. The hydrophobic pocket of the COX enzyme consists of Ala527, Val349, Ser530, and Leu531 residues in which the methyl group at the 2-position of INDO fits. This hydrophobic interaction has an important role in the binding of INDO to the COX enzyme [23,25]. INDO binds to the active site of COX, which prevents the interaction of the enzyme and molecules of arachidonic acid [33]. However, the precise mechanism of action and all of the INDO effects are not completely understood [25,35–37].

2.5 Pharmacokinetics

The pharmacokinetics of INDO include its absorption, distribution, metabolism, and excretion from the body.

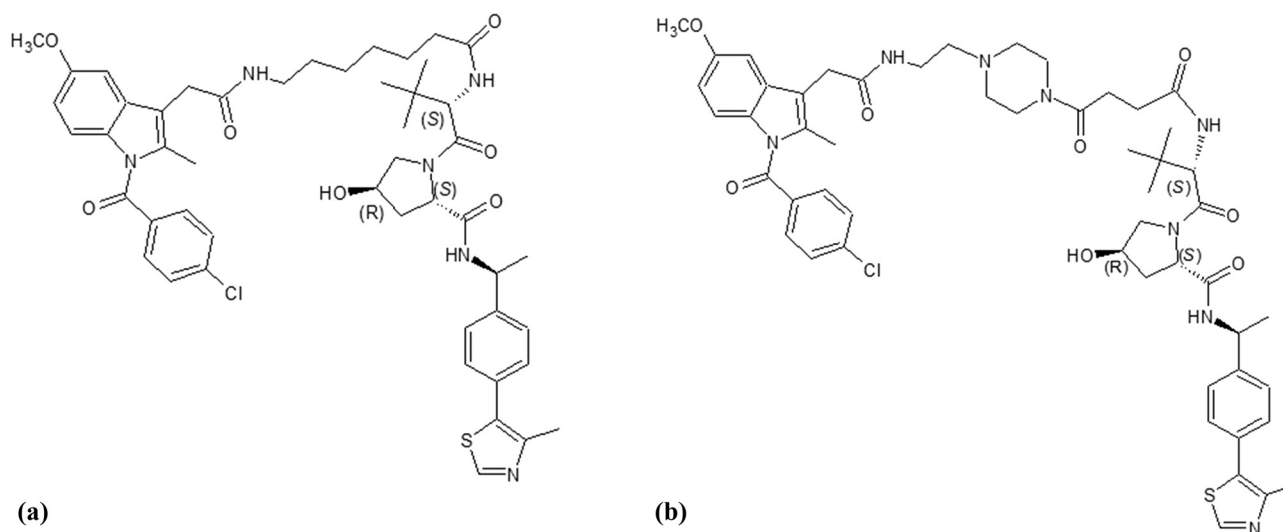


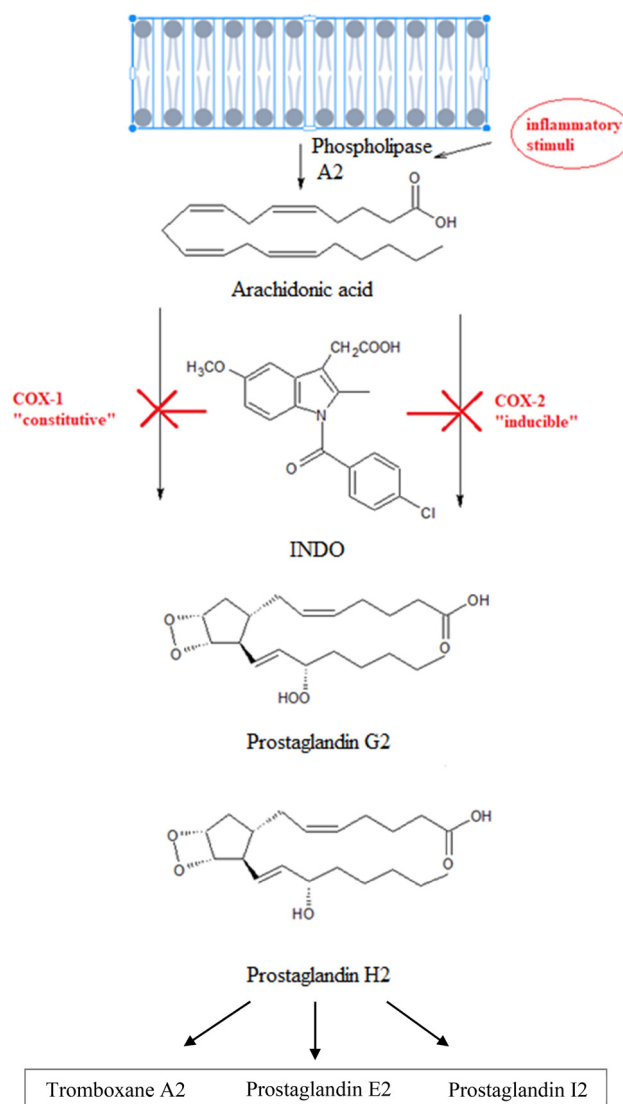
Figure 2: Structural formula of (a) **8** and (b) **9**.

It follows linear pharmacokinetics, which means that its concentration in plasma is dose proportional. INDO is rapidly absorbed from the gastrointestinal tract. Following oral administration, the bioavailability of INDO is almost 100% [38]. INDO is a weak organic acid; thus, it is 90% bound to proteins in plasma [39–41]. It can rapidly cross the blood–brain barrier and the placenta [42]. The metabolism of INDO occurs in the liver by conjugation with glucuronic acid, *O*-desmethylation, and *N*-deacylation. The resulting metabolites have no inflammatory activity. INDO and its metabolites are excreted in urine (approximately 60%), bile, and feces (approximately 34.5%) [1,43].

2.6 Application, adverse effects, and drug interactions

INDO is an NSAID with antipyretic and analgesic properties. Its pharmacodynamic effects involve inhibiting the synthesis of factors that are important for inflammation, fever, and pain, and it can be used effectively for the treatment of rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, acute painful shoulder, Alzheimer's disease, etc. [21,44]. Regardless of its benefits, INDO has several serious adverse effects, such as cardiovascular disorders (heart attack, stroke, arrhythmia, etc.), negative gastrointestinal effects (nausea, bleeding, ulceration, etc.), effects on the nervous system (headache, dizziness, insomnia, etc.), and other adverse effects that occur in less than 1% of patients. Also, INDO can cause allergic reactions with symptoms such as itching, rashes, sensitivity to sunlight, loss of hair, swollen face, lips, tongue, and throat. Other side effects can include neurological problems such as depression and tiredness. In addition, problems with the digestive system, including loss of appetite, vomiting, and diarrhea are reported. INDO consumption also can cause liver disorders, heart and kidney disorders, blood disorders, eye disorders, and problems with the ear including ringing in the ear and other hearing disturbances [45]. Myoclonus has been described as an adverse effect of many drugs but never with INDO. Hang Chen and coworkers described a case of INDO-induced myoclonus in a patient with hemicrania continua [46].

Taking certain antidepressants with an NSAID may cause patients to bruise or bleed easily [47]. Cyclosporine is an immunosuppressive drug, and when combined with INDO, they interact to increase renal toxicity and lethality [48]. Prednisolone is a corticosteroid used to treat allergies, blood disorders, infections, skin diseases, and prevent organ rejection after a transplant. The study of



Scheme 2: Proposed mechanism of action of INDO [34].

Kataoka and coworkers revealed that the ulcerogenic potential of INDO administered in combination with prednisolone might be related to the induction of physiological changes, such as endogenous prostaglandin deficiency, an increase in neutrophil activation, and gastric hypermotility [49].

3 Analytical methods for the determination of INDO

The determination of INDO is very important in industries and laboratories for the quality control of pharmaceuticals, bioavailability studies, and also for drug therapy monitoring. Accuracy, selectivity, repeatability, low limit

of detection (LOD), and wide measuring concentration range are some of the parameters that are required, and environmental safety is also now one of the most significant characteristics. Toxic and carcinogenic solvents and high concentrations of solvents should be avoided. Miniaturization is also the trend because it involves reduced amounts of sample and reagents, which results in a lower cost of analysis. Reducing the pretreatment of samples and decreasing the process steps leads to simpler methods with shorter analysis time. In addition to all the above, if the method does not require expensive instrumentation, it can be considered suitable, reliable, and desirable for analyte determination.

According to the analyzed literature published between 1965 and 2020, high-performance liquid chromatography (HPLC) is the most commonly used method for INDO determination, but UV-Vis spectroscopy, voltammetry, fluorimetry, immunochemical methods (IMs), chemiluminescence, liquid chromatography–mass spectrometry (LC–MS), potentiometry, gas chromatography (GC), colorimetry, sequential injection analysis (SIA), micellar electrokinetic chromatography (MEKC), and phosphorimetry are also used. The beginnings of development of each method for INDO

determination are chronologically listed in Figure 3. It can be seen that fluorimetry was the first method developed and used for INDO determination. Considering the requirements of the modern analytical methods, methods developed for INDO determination between 2000 and 2020 are presented in Table 1.

3.1 Colorimetry

Colorimetry is a simple instrumental quantitative technique used in various chemical analyses to determine the particular compound concentration in colored solution based on measuring color intensity. Although it is not very popular in scientific papers based on INDO determination, it was used to determine INDO for a short period of time between 1990 and 2006 [50–52].

Adegoke et al. [52] in 2006 applied colorimetry to determine INDO based on the diazo coupling reaction between INDO and the highly reactive diazonium ion, 4-carboxyl-2,6-dinitrobenzene. After dissolving INDO in glacial acetic acid, the solution was mixed with diazonium

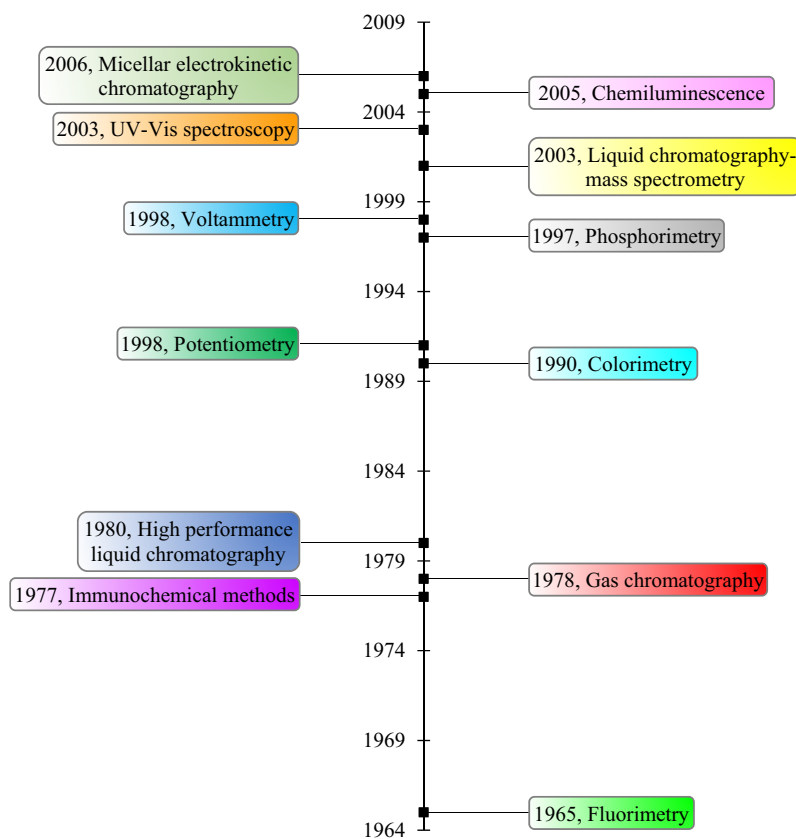


Figure 3: Chronological overview of the beginnings of development of methods for INDO determination.

Table 1: Analytical methods for determination of INDO

Method	Sample	Conditions and characteristic method parameters	Measuring range ($\mu\text{g}\cdot\text{mL}^{-1}$)	Correlation coefficient	LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery (%)	Reference
Colorimetry	INDO capsules	Max. absorption: 470 nm; temperature: 30°C (5 min), 50°C (20 min); and solvent: glacial acetic acid	3.30–11.00	0.9974	0.90	94.0–102.1	[52]
UV-Vis spectroscopy	Pharm. prep.	Max. absorption: 510 nm; temperature: 30°C; and solvent: NaOH (1 M)	0.20–10.00	0.9965	0.16	99.5–100.3	[53]
UV-Vis spectroscopy	Pharm. prep.	Max. absorption: 284.65 nm; solvent: methanol	11.88–35.64	0.9995	6.00	80.0–120.0	[54]
UV-Vis spectroscopy	Pharm. prep.	Max. absorption: 320 nm; solvent: methanol; and hydrotropic agent: niacinamide (2 M)	10.00–50.00	0.9990	0.02	99.3–101.0	[55]
UV-Vis spectroscopy	Pharm. prep.	Max. absorption: 228 nm and solvent: KOH (0.1 M)	1.00–10.00	0.9949	0.13	99.8–100.2	[56]
UV-Vis spectroscopy	Pharm. prep.	Max. absorption: 235 nm and solvent: NaOH (0.1 M)	5.00–25.00	0.9990	0.38	99.8–100.5	[57]
UV-Vis spectroscopy ¹	Pharm. prep.	Max. absorption: 320 nm and hydrotropic mixture: sodium caprylate (10%), sodium benzoate (10%), and niacinamide (10%)	10.00–50.00	0.9990	0.21	101.5–101.0	[58]
UV-Vis spectroscopy	Pharm. prep.	Max. absorption: 320 nm and hydrotropic mixture: sodium acetate (5%), sodium citrate (5%), and urea (10%)	10.00–50.00	0.9980	0.34	—	[59]
UV-Vis spectroscopy ¹	Pharm. prep.	Max. absorption: 266 nm and solvent: ethanol (50%)	1.00–6.00	0.9990	0.17	80.0–120.0	[60]
UV-Vis spectroscopy	Pharm. prep.	Max. absorption: 319 nm and solvent: NaOH (0.5 M)	10.00–60.00	0.9999	—	99.7–100.2	[61]
Fluorimetry combined with SIA	Pharm. prep.	Solvent: NaOH (0.1 M) and hexadecyltrimethylammonium bromide (20 mM); FR: 0.8 mL·min ⁻¹ ; and Ex: 278 nm, Em: 358 nm	Up to 3.58	—	5.72×10^{-3}	—	[65]
Fluorimetry combined with SIA	Human urine, Pharm. prep.	Solvent: methanol (40%); FR: 1.5 mL·min ⁻¹ ; and Ex: 283 nm, Em: 371 nm	5.50×10^{-4} to 6.50 $\times 10^{-3}$	0.9986	1.50×10^{-4}	94.0–107.0	[66]
Fluorimetry combined with SIA	Pharm. prep.	Solvent: methanol and phosphate buffer (pH 7.2); FR: 1.0 mL·min ⁻¹ ; Ex: 278 nm, Em: 378 nm	1.47–32.20	—	0.44	—	[67]
Fluorimetry combined with FIA	Pharm. prep.	Solvent: NaOH (0.1 M); FR: 1.7 mL·min ⁻¹ ; Ex: 184.9 nm, Em: 253.7 nm	3.58–1788.94	0.9952	1.79	—	[68]
Phosphorimetry	Pure INDO solution	Solvent: NaOH (0.05 M); Ex: 297 nm, Em: 450 nm	0.04–357.79	—	0.04	99.1	[70]
Chemiluminescence	Human urine	Solvent: HCl (0.1 M); MIP column: 15 mm $\times$ 4 mm; monomer: methacrylic acid; adsorption time: 50 s, FR: 1.5 mL·min ⁻¹ , washing time: 80 s with formaldehyde; reaction time: 30 s	0.10–10.00	0.9940	0.04	97.5–103.0	[71]

(continued)

Table 1: (continued)

Method	Sample	Conditions and characteristic method parameters	Measuring range ($\mu\text{g}\cdot\text{mL}^{-1}$)	Correlation coefficient	LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery (%)	Reference
Chemiluminescence combined with SIA	Pharm. prep.	Solvent: ethanol (50%); FR: $100\text{ }\mu\text{L}\cdot\text{s}^{-1}$ (detection), $60\text{ }\mu\text{L}\cdot\text{s}^{-1}$ (aspiration); aspirated solution: Ce(IV) (15 mM) in H_2SO_4 (50 mM), tris (2,2'-bipyridyl)ruthenium(II) (0.5 mM), and sodium acetate (0.4 mM), INDO	0.04–17.89	0.9997	0.02	97.3–99.6	[72]
Chemiluminescence	Water samples, human plasma	Solution: sulfur and nitrogen codoped carbon quantum dots, H_3PO_4 (4 M), INDO, and KMnO_4 ($5\times 10^{-5}\text{ M}$)	0.10–1.50	0.9982	0.06	97.8–107.0	[73]
GC-MS	Human serum and plasma	Solvent: methanol; MP: nitrogen; column pressure: 34473.79 Pa; column: zebron ZB-1 ($15\text{ m}\times 0.25\text{ mm}\times 0.25\text{ }\mu\text{m}$); t_R : 8.8 min; vaporization temperature: 40°C ; and electron impact mode, 280°C	0.25–10.00	>0.9900	0.25	80–120	[79]
HPLC	Equine serum	Solvent: methanol; MP: acetonitrile–water (51:49); FR: $1\text{ mL}\cdot\text{min}^{-1}$; column: C_{18} , Beckman ($250\text{ mm}\times 4.6\text{ mm}$) with $5\text{ }\mu\text{m}$ particles; t_R : 13.4 min; room temperature; and UV detection (254 nm)	0–5.00	0.9900	0.25	78.1	[94]
HPLC ¹	Pharm. prep.	Solvent: methanol; MP: acetonitrile–phosphoric acid (50:50); FR: 0.6, $1.2\text{ mL}\cdot\text{min}^{-1}$; columns: Zorbax SB-Phenyl ($75\text{ mm}\times 4.6\text{ mm}$) with $3.5\text{ }\mu\text{m}$ particles and Zorbax SB-CN ($150\text{ mm}\times 4.6\text{ mm}$) with $5\text{ }\mu\text{m}$ particles; t_R : 3.6 min; temperature: 25°C ; and UV detection (237 nm)	100.00–500.00	0.9999	–	95.1–100.4	[95]
HPLC	Porcine plasma	Solvent: methanol; MP: 60% acetonitrile in 0.02 M sodium acetate buffer, adjusted to pH 3.6 using orthophosphoric acid; FR: $1\text{ mL}\cdot\text{min}^{-1}$; column: C_{18} Res Elut reversed-phase column ($150\text{ mm}\times 4.6\text{ mm}$) with $5\text{ }\mu\text{m}$ particles; t_R : 3.6 min; room temperature; and UV detection (320 nm)	0.05–3.00	>0.9800	0.01	<97.0	[96]
HPLC	Plasma of premature neonates	Solvent: methanol; MP: methanol–water–orthophosphoric acid (70:29.5:0.5); FR: $1.5\text{ mL}\cdot\text{min}^{-1}$; column: Hypersil ODS C_{18} column ($125\text{ mm}\times 4\text{ mm}$) with $5\text{ }\mu\text{m}$ particles; t_R : 2.9 min; temperature: 20°C ; and UV detection (270 nm)	0.025–2.500	>0.9990	0.005	113.3	[97]

(continued)

Table 1: (continued)

Method	Sample	Conditions and characteristic method parameters	Measuring range ($\mu\text{g}\cdot\text{mL}^{-1}$)	Correlation coefficient	LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery (%)	Reference
HPLC	Human urine, Pharm. prep.	Solvent: methanol; MP: methanol–water–acetic acid (67:33:0.1); FR: $1\text{ mL}\cdot\text{min}^{-1}$; column: nucleosil RP- C_{18} (250 mm $\times$ 4.6 mm) with 5 μm particles; t_R : 7.2 min; temperature: 20°C ; chemiluminescence detection	0.01–10.00	0.9991	0.008	97.5	[98]
HPLC	Human plasma	Solvent: ethanol (96%); MP: acetonitrile–water (63:37), adjusted to pH 2 with orthophosphoric acid; FR: $0.8\text{ mL}\cdot\text{min}^{-1}$; and column: silica gel column Prodigy RP C_{18} (150 mm $\times$ 4.6 mm) with 5 μm particles, equipped with 0.5 mm prefilter and a guard column ODS C_{18} ; t_R : 6.27 min; temperature: 20°C ; UV detection (270 nm)	0.01–5.00 (total INDO), 0.01–0.20 (free INDO)	0.9995	0.003	99.6	[99]
HPLC	Pharm. prep.	Solvent: MP; MP: orthophosphoric acid–methanol–acetonitrile (40:20:40); FR: $2\text{ mL}\cdot\text{min}^{-1}$; column: LiChrosorb C_{18} (250 mm $\times$ 4.6 mm) with 5 μm particles; t_R : 7.42 min; room temperature; and UV detection (240 nm)	25.00–200.00	0.9999	0.05	99.7	[100]
HPLC ¹	Pharm. prep.	Solvent: methanol; MP: sodium phosphate buffer, pH 7.0 and acetonitrile (35:75); FR: $1\text{ mL}\cdot\text{min}^{-1}$; column: LiChrosorb C_8 (250 mm $\times$ 4.6 mm) with 5 μm particles; t_R : 6.96 min; room temperature; and UV detection (230 nm)	2.50–20.00	0.9998	0.10	99.5	[101]
HPLC ¹	Rabbit blood plasma	Solvent: methanol; MP: 0.8 mL phosphoric acid mixed with 600 mL methanol and volume made up to 1,000 mL with water; FR: $1.5\text{ mL}\cdot\text{min}^{-1}$; column: Hypersil BDS C_{18} (250 mm $\times$ 4.6 mm) with 5 μm particles; t_R : 13.83 min; temperature: 24°C ; and UV detection (240 nm)	0.25–5.00	0.9998	0.06	93.4	[102]
HPLC ¹	Pharm. prep.	MP: ethyl acetate; FR: $1\text{ mL}\cdot\text{min}^{-1}$; column: Lichrosphere RP C_8 (250 mm $\times$ 4 mm) with 5 μm particles; t_R : 2.44 min; room temperature; and UV detection (318 nm)	0.60–100.00	0.9990	0.05	98.1–101.8	[103]

(continued)

Table 1: (continued)

Method	Sample	Conditions and characteristic method parameters	Measuring range ($\mu\text{g}\cdot\text{mL}^{-1}$)	Correlation coefficient	LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery (%)	Reference
HPLC ¹	Pharm. prep.	Solvent: MP; MP: methanol–orthophosphoric acid (70:30); FR: 1.5 mL·min ⁻¹ ; column: luna C ₁₈ (2) 100 R (250 mm × 4.6 mm) with 5 μm particles; t_R : 11.29 min; and room temperature; UV detection (254 nm)	1.00–500.00	0.9997	2.10×10^{-3}	99.2–100.7	[104]
HPLC	Rabbit plasma	Solvent: MP; MP: acetonitrile–phosphate buffer, pH 4.6 (35: 65); FR: 1 mL·min ⁻¹ ; column: kromosil C ₁₈ (250 mm × 4.6 mm) with 5 μm particles; t_R : 3.31 min; temperature: 30°C; UV detection (260 nm)	20.00–200.00	0.9970	—	51.0	[105]
HPLC	Human serum and plasma	Solvent: methanol; MP: methanol–water (gradient), pH 4.5; FR: 1 mL·min ⁻¹ ; column: C ₁₈ (250 mm × 4.6 mm) with 5 μm particles; t_R : <3.5 min; temperature: 25°C; UV detection (254 nm)	5.00×10^{-3} to 0.50	—	2.18×10^{-3}	92.0–99.0	[106]
HPLC	Pure mixture of drugs	Solvent: methanol; MP: acetonitrile–phosphate buffer (65:35); FR: 1 mL·min ⁻¹ ; column: gemini RP-C ₁₈ column (250 mm × 4.6 mm) with 5 μm particles equipped with gemini C ₁₈ precolumn; room temperature; UV detection (320 nm)	—	—	—	—	[107]
HPLC ¹	Pharm. prep.	Solvent: methanol; MP: methanol–acetonitrile–sodium acetate buffer pH 3 (10:50:40); FR: 1 mL·min ⁻¹ ; column: Zorbax Eclipse Plus C ₁₈ (100 mm × 4.6 mm) with 3.5 μm particles; t_R : 3.767 min; room temperature; UV photo diode array detection (254 nm)	25.00–70.00	0.9992	1.04	80.0–120.0	[108]
HPLC ¹	Pharm. prep.	Solvent: methanol; MP: acetonitrile–sodium acetate buffer, pH 5 (60:40); FR: 1.4 mL·min ⁻¹ ; column: XTERRA® MS C ₁₈ (250 mm × 4.6 mm) with 5 μm particles; t_R : 7.751 min; temperature: 25°C; UV detection (275 nm)	10.00–100.00	0.9969–0.9983	1.07×10^{-6}	100.0	[109]
LC–MS	Pharm. prep.	Solvent: methanol; MP: acetonitrile and 20 mM ammonium acetate solution (5:1), pH 7.4; FR: 1 mL·min ⁻¹ ; column: Shimpack GLC-CN (150 mm × 4 mm) with 5 μm particles; t_R : <5 min; vaporization temperature: 200°C; detection: m/z 355.8; APCI; negative ion mode; 430–450°C	0.10–0.50	>0.9993	0.004	101.3	[110]

(continued)

Table 1: (continued)

Method	Sample	Conditions and characteristic method parameters	Measuring range ($\mu\text{g}\cdot\text{mL}^{-1}$)	Correlation coefficient	LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery (%)	Reference
LC-MS/MS ²	Rabbit plasma and uterine tissue	Solvent: methanol; MP: methanol-acetonitrile-water-formic acid (45:45:10:0.5); FR: 0.5 mL·min ⁻¹ ; column: diamonsil C ₁₈ (150 mm × 4.6 mm) with a Phenomenex C ₁₈ guard cartridge (4 mm × 3 mm), both with 5 μm particles; temperature: 20°C; detection: m/z 358 → 111; APCL; positive ion mode; 450°C	2.00×10^{-3} to 0.40 (plasma), 4.00×10^{-3} to 0.80 (uterine tissue)	0.9925	0.002	90.8	[111]
LC-MS/MS ²	Rat plasma	Solvent: methanol; MP: formic acid-acetonitrile (25:75); FR: 0.6 mL·min ⁻¹ ; column: atlantis dC ₁₈ column (50 mm × 4.6 mm) with 3 μm particles; t_R : 1.79 min; room temperature; detection: m/z 357.7 → 139.1; ESI; positive ion mode; 400°C	5.10×10^{-4} to 0.03	0.9900	5.10×10^{-4}	94.2	[112]
LC-MS ²	Human plasma and urine	Solvent: methanol; MP: formic acid-acetonitrile (47:53); FR: 1 mL·min ⁻¹ ; column: waters symmetry C ₁₈ column (150 mm × 4.6 mm) with 5 μm particles fitted with a Phenomenex C ₁₈ guard column (4 mm × 3 mm); t_R : 8.6 min; detection: m/z 358 → 139; ESI; positive ion mode; 200°C	0.02–2.97 (plasma), 0.01–4.21 (urine)	>0.9900 (plasma), >0.9800 (urine)	3.71×10^{-3} (plasma), 2.63×10^{-3} (urine)	94.0–104.0	[113]
MEKC	Human plasma	carrier: tris buffer, pH 8 with sodium octanesulfonate; uncoated fused-silica capillary (312 mm × 0.075 mm); separation voltage: 10 kV; temperature: 25°C; UV detection (254 nm, cathode at the detection side), μE : $-1.76 \times 10^{-4} \text{ cm}^2\cdot\text{V}^{-1}\cdot\text{s}^{-1}$; migration time: 5.94 min	0.30–10.00	0.9990	0.10	96.8–103.3	[115]
DPV	Pharm. prep.	Solvent: acetonitrile; working HMDE (0.4 mm ²); reference electrode: Ag/AgCl; auxiliary electrode: platinum wire; potential range: -0.903 to -1.6 V; scan rate: 10 mV·s ⁻¹ ; pulse amplitude: -50 mV; pulse time: 40 ms	0.44–3.50	—	—	—	[118]
DPV	Pharm. prep. and human plasma	Solvent: water; working MWCNT-IL modified carbon ceramic electrode, reference electrode: saturated calomel; auxiliary electrode: platinum wire; potential range: 0–0.9 V; scan rate: 50 mV·s ⁻¹	0.36–17.89	0.9900	0.09	96.0–98.0 (pharm. prep.), 97.0–99.6 (human plasma)	[119]

(continued)

Table 1: (continued)

Method	Sample	Conditions and characteristic method parameters	Measuring range ($\mu\text{g}\cdot\text{mL}^{-1}$)	Correlation coefficient	LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery (%)	Reference
DPV	Pharm. prep. and human plasma	Solvent: water; working MWCNT-IL modified carbon ceramic electrode, reference electrode: saturated calomel; auxiliary electrode: platinum wire; potential range: 0–0.9 V; scan rate: 50 $\text{mV}\cdot\text{s}^{-1}$	0.36–17.89	0.9980	0.09	97.8–101.6 (pharm. prep.), 97.9–101.0 (human plasma)	[120]
SWV	Pharm. prep., human urine and blood plasma	Solvent: water; working GCE modified with gold nanorods-graphene oxide nanocomposite incorporated carbon nanotube paste; reference electrode: Ag/AgCl; auxiliary electrode: platinum wire; potential range: 0.2–0.9 V; scan rate: 100 $\text{mV}\cdot\text{s}^{-1}$; pulse amplitude: –50 mV; pulse time: 40 ms	0.07–0.32 0.89–32.74	0.9890	6.08×10^{-3}	98.2–103.5 (humane urine and blood plasma)	[121]
DPV	Pharm. prep., human urine and blood serum	Solvent: water with alkali; working GCE modified with MWCNTs; reference electrode: Ag/AgCl; auxiliary electrode: platinum wire; potential range: 0.2–1.4 V; scan rate: 50 $\text{mV}\cdot\text{s}^{-1}$	0.07–2.15	0.9870	4.72×10^{-3}	99.3–103.4 (pharm. prep.), 97.5–102.6 (human urine), 99.5–100.5 (blood serum)	[122]
DPV	Human urine and blood serum	Solvent: phosphate buffer (0.1 M); working GCE modified with MWCNTs, NHNPs, and MCM-41 molecular sieve; reference electrode: Ag/AgCl; auxiliary electrode: platinum wire; potential range: –0.1–0.9 V; scan rate: 10–100 $\text{mV}\cdot\text{s}^{-1}$	0.29–14.31 21.47–57.25	0.9930 0.9960	0.11	94.6–106.2 (human urine), 93.7–104.0 (blood serum)	[123]
DPV	Human urine and blood serum	Solvent: phosphate buffer (0.1 M); working gold electrode modified with CdSNPs and MWCNTs; reference electrode: Ag/AgCl; auxiliary electrode: —; potential range: 0–0.8 V; scan rate: 180–600 $\text{mV}\cdot\text{s}^{-1}$	0.72–28.62	0.9980	0.17	98.2–108.2 (urine), 97.6–105.6 (blood serum)	[124]
DPV	Pharm. prep.	Solvent: water; working GCE modified with Gr-NiO; reference electrode: saturated calomel; auxiliary electrode: platinum wire; potential range: 0.6–1.1 V; scan rate: 100 $\text{mV}\cdot\text{s}^{-1}$; amplitude: 0.05 V; pulse width: 0.05 s; pulse period: 0.5 s	0.07–25.05	0.9961–0.9995	0.02	99.1–100.7	[125]
Differential pulse polarography ²	rat plasma	Solvent: methanol and dimethyl formamide; working HMDE (9 mm^2); reference electrode: Ag/AgCl; auxiliary electrode: platinum wire; potential range: from 0 to –1.6 V; scan rate: 59.5 $\text{mV}\cdot\text{s}^{-1}$; pulse amplitude: 0.1 V; pulse time: 0.01 s	0.20–1.20	0.9986	—	91.5–115.2	[126]

(continued)

Table 1: (continued)

Method	Sample	Conditions and characteristic method parameters	Measuring range ($\mu\text{g}\cdot\text{mL}^{-1}$)	Correlation coefficient	LOD ($\mu\text{g}\cdot\text{mL}^{-1}$)	Recovery (%)	Reference
DPV	Human plasma	Solvent: water with alkali; working carbon ionic liquid electrode modified with TiO_2 nanoparticles; reference electrode: Ag/AgCl; auxiliary electrode: platinum wire; potential range: 0–1 V; scan rate: $100\text{ mV}\cdot\text{s}^{-1}$	0.04–35.78	0.9911	7.50×10^{-3}	95.5–100.8	[127]
DPV	Pharm. prep.	Solvent: methanol; working boron-doped diamond electrode; reference electrode: Ag/AgCl; auxiliary electrode: platinum plate; potential range: -0.5 – -1.3 V ; scan rate: $10\text{ mV}\cdot\text{s}^{-1}$; pulse amplitude: 50 mV ; pulse time: 20 ms ; potential step: 5 mV	0.18–35.78	0.9850–0.9980	0.04	95.0–103.2	[128]
Potentiometry	Pure mixture of drugs	Solvent: acetonitrile; ISE: glass electrode; reference electrode: Ag/AgCl	—	0.9944	—	—	[130]
Potentiometry	Pharm. prep.	Solvent: ethanol; ISE: liquid membrane (ion-pair: rhodamine B-INDO; plasticizer: dibutyl phthalate; PVC; solvent: cyclohexanone); reference electrode: Ag/AgCl; slope: $60\text{ mV}\cdot\text{decade}^{-1}$; response time: 3 – 5 s	35.78–17889.40	—	10.73	97.2–99.7	[131]
Potentiometry	Pharm. prep.	ISE: liquid membrane (ion-pair: tetraoctylammonium 1-(<i>p</i> -chlorobenzoyl)5-methoxy-2-methyl-3-indolylacetate; plasticizer: dibutyl phthalate; PVC; solvent: tetrahydrofuran); reference electrode: Ag/AgCl; slope: $59.8\text{ mV}\cdot\text{decade}^{-1}$; response time: 12 s	3.58–3577.88	0.9980	1.13	—	[132]
ELISA	Water	Solvent: methanol; conjugate preparation: anhydride ester method; conjugate: goat anti-rabbit IgG-horseradish peroxidase; absorption: 450 nm	1.00×10^{-5} to 0.01	0.9980	1.00×10^{-5}	98.0–106.0	[135]
ELISA	Human serum	Conjugates: INDO-keyhole limpet hemocyanin; INDO-ovalbumin, INDO-horseradish peroxidase; conjugate preparation: <i>N</i> -hydroxysuccinimide active ester procedure; absorption: 450 nm	0.01–2.00	—	0.02 (ovalbumin format); 0.01 (horseradish peroxidase format)	82.0	[136]

Pharm. prep., pharmaceutical preparations; Em, emission; Ex, excitation; FR, flow rate; MP, mobile phase; t_R , retention time; and μ_E , electrophoretic mobility.

¹ Methods declared as validated in accordance with ICH guideline bioanalytical method validation M10 [137]; ² Methods declared as validated in accordance with bioanalytical method validation guidance for industry [138].

ion, used as a chromogenic derivatizing reagent. The incubation was performed at 30°C (5 min) and 50°C (20 min). The resulting orange azo compound, extracted with ethyl acetate, was stable for 3 h, with a maximum absorption at 470 nm.

Although colorimetry is a fast, simple, low-cost, and portable system that can easily be transported, it has a few very important disadvantages, such as the impossibility of determination of colorless compounds and low sensitivity. Also, the accuracy of the method can easily be affected with the same color interferences and matrix interferences in uncontrolled conditions. Considering these limitations, other analytical methods are more convenient for INDO determination.

3.2 UV-Vis spectroscopy

UV-Vis spectroscopy is an analytical method for qualitative and quantitative determination of compounds containing chromophores that absorb characteristic wavelengths of ultraviolet (UV) or visible light. According to the Beer–Lambert law, absorption is related to the concentration of the sample. Also, this method is frequently used as a starting point of various research, which gives preliminary data about the absorption characteristics of a target analyte. UV-Vis spectroscopy is one of the simplest methods used in the determination of INDO. In addition to simplicity, it is characterized by simple sample preparation, low cost, and relatively short analysis time. Sample preparation for INDO determination using UV-Vis spectroscopy includes dissolving in an alkali-based solvent, methanol, ethanol, or hydrotropic solution, at room temperature, and filtration. UV-Vis spectroscopy used for INDO determination appeared in 2003, and it is still used today.

Determination of INDO using UV-Vis spectroscopy began when Nagaraja et al. [53] developed a sensitive method for the determination of this drug from a capsule formulation. The basis of the method is the coupling reaction of INDO with a diazotized *p*-phenylenediamine dihydrochloride in sulfuric acid medium, giving a red product with a maximum absorption at 510 nm that was stable for 20 h.

A year later, a new study described the direct analysis of INDO and its degradation product (impurity) 5-methoxy-2-methyl-3-indoleacetic acid, using derivative spectroscopy [54]. A zero-order spectrum was recorded at a wavelength range between 200 and 400 nm and converted to the corresponding derivatives. The value of the first derivative for INDO was determined by using the zero-crossing method

and read at 284.65 nm. The LOD indicated that the developed method was sufficiently sensitive and, as such, could detect impurities at levels of about 0.2%, which corresponds to pharmacopoeial requirements.

A slightly different method emerged in 2011 when Maheshwari et al. [55] developed a more environmentally friendly spectroscopy using a hydrotropic solubilization procedure to dissolve the sparingly soluble INDO with the addition of niacinamide as a hydrotropic agent. It had no effect on the overall analysis or on environmental pollution, and there was a fivefold increase in the solubility of INDO compared to its solubility in water.

Ali et al. [56] developed a very simple, fast, and precise method for the UV-Vis spectroscopic determination of INDO that can be applied in routine pharmaceutical analyses. KOH was used for the decomposition of INDO to *p*-chlorobenzoic acid and 5-methoxy-2-methyl-3-indoleacetic acid. It was concluded that this method could be applied directly for the estimation of *p*-chlorobenzoic acid and indirectly to estimate the INDO content in pharmaceutical formulations.

Rathod et al. [57] developed a simple UV spectrophotometric method for INDO determination in bulk drug and capsule formulation, with the maximum absorbance of INDO at 235 nm. NaOH (0.1 M) was used for solubilization of INDO and the method was validated.

In the same year, Jain et al. [58] developed a sensitive, simple, and nontoxic UV spectroscopic method for the determination of INDO in pharmaceutical formulations. As in previous research, the importance was on choosing an environmentally friendly solvent, so they developed a mixed solvency concept using sodium caprylate, sodium benzoate, and niacinamide. By applying this type of concept, an innumerable solvent system could be developed to prevent the use of organic solvents that are major pollutants.

In 2018, Rathod et al. [59] developed another precise, accurate, and fast UV-Vis spectroscopy method for the determination of INDO in pure form and in pharmaceutical formulations. The principle was almost the same as in previous research, but they used a different mixed hydrotropic solution (sodium acetate, sodium citrate, and urea), which had an important effect on the final measurement results. The solvent used did not have any negative impact on the environment.

In 2019, another study was published for the simple determination of INDO in pharmaceutical formulations using UV spectroscopy and ethanol as a solvent [60]. The absorbance was measured at 266 nm.

According to the latest research (2020), it can be seen that spectroscopic methods are still used for INDO

determination, using the same principles but with numerous method modifications. Abed et al. [61] proposed a very simple, accurate, and precise green analytical chemical method to measure INDO as a copper complex using UV-Vis spectroscopy. The absorbance of the complex was measured at 319 nm. The copper was used because it is a trace metal that has a very important role in the creation of drug complexes that are easily recognizable in *in vitro/vivo* studies [62]. This method represents a new application of synthesized complexes of copper and INDO, so the major innovations from this research could be used as a guide for scientists to continue this type of study.

Due to its accuracy and availability in laboratories, UV-Vis spectroscopy was frequently used for INDO determination, considering its low cost. All the analyzed samples were pharmaceutical, and unlike the colorimetry, all reactions were performed at room temperature. One of the main problems during the method development is the insolubility of INDO, which can be overcome using hydrotropic solutions. Over the years, the effort was to develop an environmentally friendly method. The method can be affected by temperature, pH, interferences, and impurities, but these limitations can be overcome by optimizing the working conditions. Considering the above, it is expected that UV-Vis spectroscopy will continue to develop and remain one of the most commonly used methods for INDO determination.

3.3 Fluorimetry

Fluorimetry is a quantitative method that has a wide range of applications. It measures the fluorescence at a characteristic emission wavelength that occurs after excitation with a photon, during relaxing back to the ground state. The development of this method for INDO determination started from early 1965 [41,63,64]. Following each other's ideas, scientists have perfected this method to the point that it can be applied in routine pharmaceutical analyses, as well as in clinical practice to monitor and determine patient therapy. Although INDO does not exhibit native fluorescence, it can easily be hydrolyzed in an alkaline medium, which leads to the possibility of fluorometric determination of formed fluorescent product. A major step forward in fluorometric determination occurred when this method was combined with other methods to obtain the most reproducible results. For example, coupling fluorimetry with SIA or flow injection analysis (FIA) leads to reduced solvent consumption and better reproducibility and accuracy, due to the minimized intervention of the operator.

In 2005, Pinto et al. [65] developed a pulsed flow SIA based on pulse-generating solenoid micropumps, instead of the conventional solution propelling units commonly used in SIA, combined with fluorometric detection. The developed method made it possible to reduce reagent consumption and waste, as well as increasing sample flow.

In 2010, Molina-García et al. [66] developed a fluorometric SIA optosensor for the determination of INDO in urine and pharmaceuticals. The authors used a solid support into the flow cell, which allowed the required selectivity and high sensitivity.

As the SIA method had proven to be very successful in previous research, Passos et al. [67] developed a method combining fluorimetry and SIA in 2011, with some modifications. A novelty was the use of UV light in a photo-reactor, which stimulated an increase of INDO fluorescence and increased the sensitivity of the method.

In 2018, a new method was developed for INDO determination using FIA and fluorometric detection [68]. The specificity of the method was the application of a homemade fluorimeter with a low-pressure mercury lamp.

Considering high sensitivity, specificity, and precision as well as very low detection limits, fluorimetry, and fluorometric detectors combined with other analytical methods are attractive for INDO determination. Most of the authors used pharmaceutical preparations as analytes. Sample preparation was simple. NaOH was the most commonly used for hydrolysis of INDO. The novelty was the introduction of cationic surfactant, which catalyzes the alkaline hydrolysis and leads to increased fluorescence intensity. The difference in biological sample preparation is the addition of acetonitrile as a typical chaotropic reagent. During the method development, attention should be paid to pH, temperature, oxygen, and heavy metals that can affect the fluorescence. Also, colored compounds present in solutions that contain fluorescence compounds, can absorb fluorescence, thus acting as internal filters. As fluorimetry was the first method for INDO determination and has been used for more than 50 years, it is significant for INDO determination and should undoubtedly continue to develop.

3.4 Phosphorimetry

Phosphorimetry is a quantitative analytical method based on the same principles as fluorimetry. The difference is a change in electron spin, in energy transitions, that occurs in phosphorimetry but not in fluorimetry. Consequently, the emission process is much slower in phosphorimetry than in fluorimetry. However, phosphorimetry was not

very popular for INDO determination. Only two studies using room-temperature phosphorimetry were reported, of which the first was published in 1997 [69].

Another study was reported 5 years later, in 2002 [70]. In that article, authors used room-temperature phosphorimetry on a poly(vinyl alcohol) substrate for INDO determination via its indole hydrolysis products 5-methoxy-2-methylindole-3-acetic acid and *p*-chlorobenzoic acid. INDO was easily hydrolyzed using NaOH whereby its concentration had to be as low as possible because NaOH caused background emissions. The authors tested the effect of heavy atoms on method characteristics using three inorganic salts, and they concluded that potassium iodide (KI) significantly enhanced the phosphorescence signal for 5-methoxy-2-methylindole-3-acetic acid. Also, they used sodium dodecyl sulfate for the improvement of the efficiency of the heavy atom on the poly(vinyl alcohol) substrate. It was concluded that the method using poly(vinyl alcohol) substrate is more sensitive and simple than the method using a paper substrate.

Although phosphorimetry is very sensitive method for pharmaceutical analysis, in most cases, it demands using of degassed and purified solutions and low temperatures to prevent quenching the analyte emission. The room-temperature phosphorimetry, which does not require liquid nitrogen and cryogenic equipment, could overcome some of these limitations, but still phosphorimetry is not widely used for INDO determination.

3.5 Chemiluminescence

Chemiluminescence is an analytical method based on radiation emitted during the returning of excited species to the ground state. The source of excitation is the energy obtained from chemical reactions, unlike phosphorescence and fluorescence where the source is absorbed light energy. Although chemiluminescence has wide application, it was used for INDO determination for the first time in 2005.

In 2005, Nie et al. [71] successfully developed the first chemiluminescent system for the determination of INDO in urine. The basis for their research was the reaction between INDO and soluble Mn(IV), which can produce chemiluminescence, with the use of formaldehyde to enhance this reaction. However, this method cannot be used directly for INDO determination in complex systems because of poor selectivity. Therefore, a molecular imprinted polymer (MIP) column was used as an INDO recognition element, which had the ability to separate it from the interfering substances. The INDO MIP system was combined with the chemiluminescent reaction described above, enabling greatly improved selectivity and direct analyte determination.

Two years after this research, scientists started to combine chemiluminescence with other analytical methods to obtain more reproducible results. Mervartová et al. [72] used the combination of chemiluminescence and SIA for the determination of INDO in semisolid forms (ointments and gels). SIA was acceptable because of its many advantages, such as the on-line preparation of unstable reagents and waste reduction, as well as a reduction in sample and reagent consumption. The chemiluminescent radiation was based on the reaction of INDO with tris(2,2'-bipyridyl)ruthenium(III), which was generated online in the SIA system.

The latest research came in 2017, when Hallaj et al. [73] brought this method to a new and highest level by developing a novel chemiluminescence sensor for the simple and sensitive determination of INDO in environmental water and in biological samples, based on a sulfur and nitrogen codoped carbon quantum dot–KMnO₄ reaction. Sulfur and nitrogen codoped carbon quantum dots were prepared by a simple hydrothermal method and characterized by fluorescence spectrum, transmission electron microscopy, and Fourier-transform infrared spectroscopy. The study showed that the intensity of chemiluminescence decreased in the presence of INDO because of competition for KMnO₄ between the sulfur and nitrogen codoped carbon quantum dots and the INDO.

Chemiluminescence is a highly sensitive method due to its low background signal. Considering its high selectivity, chemiluminescence does not require complicated pretreatment of the samples. In studies previously described, pharmaceutical formulations were dissolved in ethanol (50%) or HCl (0.1 M) while biological samples were prepared using trichloroacetic acid (10%), Ba(OH)₂ (0.1 M), and ZnSO₄ (0.1 M) for protein precipitation. Despite its high selectivity and low detection limits, chemiluminescence has a few disadvantages that can limit its application, such as the influence of interferences, nonlinearity, sensitivity to environmental conditions, and weak chemiluminescence. The main drawback is that chemiluminescence is applicable only for chemical reactions that produce energy in the form of photons. All of these limitations make this method less attractive for INDO determination. However, considering the only few scientific papers describing chemiluminescence as the method for INDO determination, the method has the potential to develop.

3.6 GC

Chromatographic methods are qualitative and quantitative analytical techniques based on the separation of mixture components according to differences in the rates at

which they are carried through the stationary phase by a gaseous or liquid mobile phase. These methods can be used for separation, purification, and identification of the mixture components.

GC is a chromatographic method where chemically inert gas is used as a mobile phase. It was used extensively to determine INDO in the 1970s and 1980s [74–77]. The improvement of the method was its combination with MS [78,79]. In the last article, authors reported using GC–MS for INDO determination in serum and plasma [79]. Before analysis, INDO was extracted using methylene chloride and phosphate buffer (pH 6) and derivatized using bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane.

GC was the first chromatographic method used for INDO determination. Although it requires a small amount of sample and can be used for the separation of very complicated mixtures, its application has gradually decreased. The main reason is the long-term extraction of solvents, the use of explosive esterification reagents, and the often incomplete separation from various components of body fluids. Considering these drawbacks, GC was replaced by HPLC, as the main chromatographic method for INDO determination.

3.7 HPLC

LC is a chromatographic method with the liquid solvent used as a mobile phase. In European Pharmacopoeia [18], British Pharmacopoeia [19], and United States Pharmacopoeia [20], LC with UV detection was described as a method for INDO determination. In European and British Pharmacopoeia, acetic acid ($10 \text{ g}\cdot\text{L}^{-1}$) and acetic acid ($10 \text{ g}\cdot\text{L}^{-1}$) in acetonitrile, in gradient mode, were used as a mobile phase. The absorbance was measured at 254 nm, at a temperature of 50°C , and flow rate $0.8 \text{ mL}\cdot\text{min}^{-1}$, using a column ($100 \text{ mm} \times 4.6 \text{ mm}$) with octadecylsilyl silica ($2.6 \mu\text{m}$ particle size). In United States Pharmacopoeia, formic acid (0.1%) and acetonitrile (55:45) were used as a mobile phase. The absorbance was measured at 240 nm, at a temperature of 30°C , and flow rate $1.5 \text{ mL}\cdot\text{min}^{-1}$, using a column ($250 \text{ mm} \times 4.6 \text{ mm}$) with octadecyl silane ($5 \mu\text{m}$ particle size). Although the LC methods for INDO determination, described in pharmacopeias, are reference methods for quality control due to its high sensitivity, they are characterized with large consumption of organic solvent (acetonitrile) and use of expensive equipment. Maybe the pharmacopeial methods for INDO determination should be revised and replaced with less complicated and less demanding but equally sensitive methods.

During LC method development, it was concluded that better efficiency of chromatographic separation could be obtained using chromatographic columns with a reduced particle size of packing. For that reason, HPLC, with particle diameters as small as $3\text{--}10 \mu\text{m}$, was developed. To ensure reasonable flow rates in such systems, pressures of several million Pascal are necessary. Today, HPLC is one of the most versatile chromatographic methods that is widely used. Also, it is one of the most commonly used methods for INDO determination. The determination of INDO using HPLC began in 1980 [80]. The method continued to develop until today [81–109]. Numerous modifications and improvements have been made over the long period in which this method has been used. As a result, HPLC is now one of the most accurate methods in clinical and pharmaceutical practice. Its specificity, the small amount of sample required, and the short analysis time are the main advantages, making this method attractive, especially when a large number of samples are analyzed daily. All HPLC methods developed for the INDO determination were reverse-phase HPLC.

In 2000, Grippa *et al.* [94] determined INDO, as well as other anti-inflammatory drugs, in equine serum using an HPLC method. The choice of extraction solvent (ethyl acetate) showed high selectivity and nontoxic properties, and it was concluded that the developed method was suitable for the antidoping control of racehorses. It was also the first reported HPLC method that enabled the simultaneous determination of multiple different drugs in equine serum.

In 2005, Nováková *et al.* [95] developed a fast, simple, and accurate HPLC method to determine INDO and its degradation products (impurities), 4-chlorobenzoic acid and 5-methoxy-2-methylindoleacetic acid. Separation of the compounds was performed simultaneously and automated in one step. Different stationary phases were studied, with an emphasis on newer types of columns (Zorbax), which allowed faster separation of INDO and its impurities. The method was suitable for the analysis of INDO-based gels and for the study of degradation and stability of various pharmaceutical formulations.

In addition to detecting INDO in human plasma, Boon *et al.* [96] developed an HPLC method for INDO determination in porcine plasma. By checking the validity of the method, they concluded that the same method could be used for the determination of INDO in human plasma. Plasma proteins were precipitated and extracted using acetonitrile, and an autosampler was used for direct injection into the HPLC system. In addition to being precise, this method was fast, taking less than 3 h to extract and

analyze 20 samples. The method could have wide clinical application, especially in pediatric samples.

In 2006, Al Za'abi et al. [97] made progress in clinical practice, with the aim of developing a precise microscale HPLC method that would enable the determination of INDO from the plasma of premature neonates with patent ductus arteriosus (PDA). The developed method used a very small sample volume, minimal sample processing, and a shortened overall analysis time. It was concluded that the method was suitable for pharmacokinetic studies of INDO and the monitoring of therapy in infants with PDA.

In 2007, HPLC moved to a new level, with scientists starting to combine it with chemiluminescence and electrolytic methods to obtain more reproducible results. Zhang et al. [98] determined INDO in urine and pharmaceutical preparation using HPLC with *in situ* electrogenerated Mn(III) chemiluminescence. The basis for this method was the direct reaction of INDO and Mn(III).

In 2009, Dawidowicz et al. [99] developed a sensitive and accurate HPLC method for the determination of total and free INDO in human plasma. They emphasized the clinical importance of the method because of the possibility of determining the effectiveness of INDO during therapy, especially in neonates with PDA. However, there were some positive modifications. Sample preparation for the determination of total INDO was performed using liquid–liquid extraction, and the authors introduced separation with an ultrafiltration method on Amicon micropartition system (MPS) units to determine free INDO. This procedure allowed much better purification of samples compared to classical protein precipitation.

Tsvetkova et al. [100] developed a HPLC method for the determination of INDO and related substances in raw materials and pharmaceutical compositions. In addition to the detection of INDO, the researchers investigated the kinetics of the drug, that is, its alkaline hydrolysis, and concluded that INDO follows first-order kinetics.

As the HPLC method proved to be very successful for INDO determination in pharmaceutical applications, Ivanov et al. [101] applied it in 2013, in a similar way to previous research, to determine the concentration of INDO and paracetamol in tablet dosage forms, concluding that the method was suitable for routine pharmaceutical analyses.

In the same year, Nandy et al. [102] applied an accurate, precise, and selective HPLC method for INDO determination in animal samples (rabbit blood plasma). Liquid–liquid extraction was used for sample preparation, and the method was applicable for INDO detection in blood plasma serum.

Haq et al. [103] developed an accurate and precise HPLC method for the determination of INDO in pharmaceutical

formulations, using an ecological approach and thus ensuring the health of scientists during the experimental research. The approach was based on the use of an environmentally friendly eluent, ethyl acetate; therefore, the new method was called the green HPLC method. There were no long-lasting extraction procedures, and a very low retention time was observed. The method was applicable for routine pharmaceutical analyses.

A year later, Lariya and Agrawal [104] developed a fast, reproducible, accurate, and precise isocratic HPLC method for the determination of INDO, methotrexate, and dexamethasone. This method is very practical in clinical practice because it allows the simultaneous determination of several different drugs used in combination therapy.

In 2016, Babu et al. [105] developed and validated a bioanalytical HPLC method for the simultaneous estimation of INDO and omeprazole in rabbit plasma, with INDO being extracted by a liquid–liquid extraction method using acetonitrile. This method could also be used for pharmacokinetic studies.

The beginning of 2017 was marked by the development of a rapid and simple HPLC method for INDO determination. Chamkouri et al. [106] used ultrasound-assisted dispersive liquid–liquid microextraction for the isolation of INDO from human plasma and serum samples. One of the most important advantages of this method was the low consumption of organic solvents.

Elshorbagy et al. [107] proposed an analytical assay for INDO determination in the presence of quercetin. INDO has a high ulcerogenic potential, while quercetin can prevent gastric damage and INDO-induced ulcers; thus, they are commonly combined in therapies. Although INDO and quercetin could be determined separately using a UV assay, it had not been possible to quantify them simultaneously: the solution to this problem was the application of the HPLC method.

Pai and Sawant [108] developed and validated a simple, accurate, and precise method for the determination of INDO in pharmaceutical formulations using HPLC with a UV-VIS photo diode array detector. The two degradation impurities of INDO, 5-methoxy-2-methyl-indoleacetic acid and 4-chlorobenzoic acid, were determined in addition to INDO, with an emphasis on shortening the overall analytical analysis, considering that all the compounds eluted within 5 min. The method can also be used in routine pharmaceutical analyses.

The last study involving a HPLC method for INDO determination was reported in 2020 when Assali et al. [109] synthesized a codrug of INDO and paracetamol and combined it with famotidine, which is used to reduce gastric side effects. This precise, accurate, selective, and

robust method was validated and used to simultaneously quantifying INDO, paracetamol, famotidine, and the codrug.

Due to the low analyte concentration in the sample and possible interferences, pretreatment of the sample is crucial and differs for each sample. In described studies, biological samples were more commonly analyzed and in most of them methanol or mobile phase, used in the chromatographic system, was used as the solvent for INDO. Acetonitrile and trichloroacetic acid (1%) were most commonly used for protein precipitation. In a few articles, the organic solvent was added to a biological sample, and after the separation of the organic phase, it was evaporated to dryness in a stream of nitrogen or argon. The remaining residue was then dissolved in the mobile phase. To protect HPLC columns, samples were filtered before the analysis. Separation was mainly carried out in the C_{18} column with $150\text{ mm} \times 4.6\text{ mm}$ or 250×4.6 inner diameter and $5\text{ }\mu\text{m}$ particles. The mobile phase in most cases included acetonitrile or methanol. LOD was the lowest in separation system with acetonitrile-based mobile phase. In the studies described, UV detection was predominant. Although, HPLC is a highly precise, reproducible, and widely applicable method, it uses large amounts of organic, cancerogenic solvents. Also, HPLC systems are expensive and complex, and long-term reproducibility could be a problem due to the reduction of column performance. For that reason, the development of less complicated, less expensive and more environmentally friendly methods for INDO determination should be continued.

3.8 LC–MS

LC–MS is a powerful analytical method based on LC or HPLC with their advanced physical separation properties and MS as a highly specific mass analysis detector. This method provides good efficiency of drug detection; thus, it can be used in clinical and pharmaceutical analyses. Despite its complexity, LC–MS permits smooth utility and application. Generally, a low LOD and limit of quantitation of drugs can be achieved using LC–MS, with a high degree of specificity in a relatively short analysis time, without a need for complete chromatographic resolution of analytes.

The first article relating to the determination of INDO by the LC–MS method was published in 2001, when Abdel-Hamid *et al.* [110] applied this method to determine several NSAIDs, both in a mixture and separately. Diclofenac sodium, flufenamic acid, INDO, and ketoprofen were determined. The mass spectrometer, operated in the

single ion monitoring mode, was programmed to admit the negative ions $[M-H]^-$ at m/z 355.8, and atmospheric pressure chemical ionization (APCI) was used for ionization. This fast, highly specific, and sensitive method could be used for the routine determination of NSAIDs in biological fluids.

In 2012, Liu *et al.* [111] developed and validated a rapid and sensitive LC–MS/MS method for INDO determination in biological samples. Acetonitrile was used for the extraction of INDO from rabbit plasma and uterine tissue by simple protein precipitation. The authors applied an APCI in positive ionization mode, and quantitation was performed using selected reaction monitoring of precursor-production transitions at m/z $358 \rightarrow 111$.

In 2013, Suresh *et al.* [112] developed and validated a specific, rapid, and simple bioanalytical method for the determination of INDO in rat plasma. The authors applied LC coupled to tandem MS with electrospray ionization (ESI) in the positive-ion mode. The mass spectrum for INDO revealed a peak at m/z 357.7, as protonated molecular ions, $[M + H]^+$. For quantification of INDO in the multiple reaction monitoring mode, transition of the m/z 357.7 precursor ion to the m/z 139.1 product ion was used. The sample was prepared using liquid–liquid extraction with acetonitrile, and the developed method could be used for pharmacokinetic research in rats.

In the same year, the last study concerning the determination of INDO by LC–MS was published. Wang *et al.* [113] determined INDO in the maternal plasma and urine of pregnant patients under treatment using the deuterium-labeled isotope of INDO as an internal standard because it minimized the impact of the matrix. The sample was prepared by liquid–liquid extraction. Ionization of INDO with the ESI interface in positive and negative ion modes was also investigated. Due to its higher sensitivity, the positive ion mode was selected for the overall INDO analysis, targeting ions at m/z 139. The validated method was accurate and precise and was suitable for determining the pharmacokinetic parameters of INDO during pregnancy.

As in HPLC, preparation of the sample is necessary before the analysis. INDO detection included adding of acetonitrile or chloroform, centrifugation, evaporation of organic phase in a stream of nitrogen, and dissolving the residue in the mobile phase. The mobile phase, in all papers described, included acetonitrile. Separation was mostly carried out in the C_{18} column with $150\text{ mm} \times 4.6\text{ mm}$ inner diameter and $5\text{ }\mu\text{m}$ particles. With the reduction of column size to $50\text{ mm} \times 4.6\text{ mm}$ and particles size to $3\text{ }\mu\text{m}$, the lowest detection limit was achieved. Most of the analyzed samples were biological and INDO was always dissolved in methanol.

In MS detection, special attention should be paid to the choice of ionization method. For INDO determination, “soft” ionization methods such as ESI and APCI, which result in fewer fragments, are preferable. The molecular ion at m/z 358 proves the presence of INDO. This value can be lower because of hydrogen loss due to the ionization method. The ions at m/z 139 and 111 are the major fragmentation ions. Zhou and Gilpin [114] developed rapid ESI-MS method for examining the thermal decomposition of three pharmaceuticals including INDO. The major ions observed were a single thermal decomposition fragment (m/z 139), the MH^+ ion (m/z 358), additional parent-adduct ions $M_2NH_4^+$ (m/z 375), MNa^+ (m/z 380), MK^+ (m/z 396), and dimeric products M_2H^+ (m/z 715), $M_2NH_4^+$ (m/z 732), M_2Na^+ (m/z 737), and M_2K^+ (m/z 753). The main dissociation ion of the precursor m/z 358 ion was the m/z 139 ion that results from cleavage of the amide bond. The product ion of the m/z 139 ion is the m/z 111 ion.

Besides significant advantages of LC–MS method, such as exceptional sensitivity and specificity, LC–MS is characterized by a high cost of instrumentation and maintenance. This drawback makes LC–MS unaffordable for many laboratories, which directly affects the further development of the method.

3.9 MEKC

Although HPLC is the most commonly used method in the pharmaceutical determination of NSAID drugs, it uses too much organic solvent. The alternative could be MEKC, as a combination of electrophoretic and chromatographic basis, due to the lower cost of instrumentation and nonuse of organic cancerogenic solvents. The MEKC is a separation method based on partitioning of the analyte between the mobile phase (background electrolyte) and pseudo stationary phase (micelles of a surfactant). Micelles with the analyte migrate through the system due to the electroosmotic flow, under the electric field.

Lin et al. [115] proposed a new method in 2006, MEKC, for INDO determination in the plasma of premature infants with PDA. The analytical procedure began with the processing of plasma samples by the precipitation of proteins with acetonitrile. It was followed with centrifugation and evaporation of supernatant to dryness and dissolving in tris buffer (pH 8) containing internal standard. Although sodium dodecyl sulfate is the most commonly used surfactant in MEKC, the authors applied sodium octanesulfonate as a micellar source with tris buffer to differentiate INDO from endogenous components.

The results obtained using this method were found to give satisfactory agreement compared with those obtained using HPLC.

MEKC can separate both neutral and ionic analytes, which makes it attractive for the separation of pharmaceutical substances that are commonly neutral. However, it is not a popular method for INDO determination due to low sensitivity in the low concentration range.

3.10 Voltammetry

Voltammetry is an electroanalytical method based on measuring the current as a function of applied potential. The measurements are performed in an electrochemical cell containing three electrodes: working, reference, and auxiliary. The main advantages of this method compared to chromatography are the elimination of time-consuming solvent extraction steps, elimination of toxic organic solvents, shorter analysis time, reduced costs, and simplicity. Voltammetry is one of the most applicable methods for determination of INDO and similar drugs. The first scientific articles that described using of voltammetry for INDO determination were published in 1998 and 1999 [116,117]. The following series of articles show the progress of this method in the period from 2002 to 2020. Although each study had a roughly similar principle, there was still a very important difference. The specificity of each study was determined by a working electrode that was modified from year to year and ultimately achieved very positive changes, thus making this method very sensitive, precise, and accurate. Most of the research used cyclic voltammetry (CV) for electrode characterization and differential pulse voltammetry (DPV) for INDO quantification.

In 2002, Reguera et al. [118] proposed method where they applied the partial least squares calibration method to determine several anti-inflammatory drugs, including INDO. The method was very advanced because it enabled the separation of analytes from a complex mixture, even where analyte signals overlap and the classical voltammetric approach does not allow a single-drug analysis. A hanging mercury drop electrode (HMDE) was used as a working electrode.

Sarhangzadeh et al. [119] returned to a more classical voltammetric determination of INDO. They applied DPV to identify INDO in pharmaceutical preparations and human plasma. The specificity of the method relates to the application of a carbon ceramic working electrode modified with a multiwalled carbon nanotube and ionic liquid (MWCNT–IL). Carbon nanotubes have specific properties, such as high stability and good electrical conductivity.

Furthermore, ionic liquids are characterized by high thermal and chemical stability, negligible vapor pressure, and high ionic conductivity. By combining these materials, an increase in the electrochemical response of electroactive substrates was obtained. Sarhangzadeh *et al.* [120] also reported very similar research with very similar results using the same electrode, with the simultaneous determination of INDO and diclofenac.

Arvand and Gholizadeh [121] developed square wave voltammetry (SWV) for the determination of INDO in human blood serum, urine, and pharmaceutical preparations. The drug was determined on a glassy carbon electrode (GCE) modified with a gold nanorods-graphene oxide nanocomposite incorporated carbon nanotube paste. This type of electrode showed excellent electrochemical catalytic activity toward INDO oxidation, more reactive sites, and a high effective surface area.

In 2014, Sataraddi *et al.* [122] applied CV and DPV for INDO determination using GCE modified with MWCNTs. The scientists applied CV to define the electrochemical behavior of INDO and to find peaks. They chose this type of working electrode to improve the oxidation of INDO in a slightly acidic solution, which led to increased current intensity and the formation of two anode peaks and one cathode peak. The concentration of INDO in pharmaceutical preparations, human blood serum, and urine was then determined by DPV.

In 2015, Babaei *et al.* [123] developed a sensitive method for determining three drugs, including INDO. As a working electrode, they used GCE modified with MWCNTs, nickel(II) hydroxide nanoparticles (NHNPs), and a mobil composition of matter No. 41 (MCM-41) molecular sieve. CV was applied to test the effect of the composition of MWCNTs, NHNPs, and MCM-41 as a GCE modifiers, whereas DPV was used for the simultaneous determination of the INDO, dopamine, and acetaminophen concentrations in urine and serum samples. The applied modification of the GCE led to excellent electrocatalytic performance for the simultaneous determination of the tested drugs. In addition, the fabrication procedure of the electrode was simple, and no sample pretreatments or time-consuming extractions were necessary. The developed method was very successful and was relevant for practical applications. Babaei and Yousefi [124] continued their work in 2020, when they proposed a gold electrode modified with cadmium sulfide nanoparticles (CdSNPs) and MWCNTs for the simultaneous determination of INDO, uric acid, and norepinephrine in human urine and blood serum. Again, they used a combination of methods: CV, DPV, and chronoamperometry, whereas the electrochemical behavior of the mixture and the analyte concentrations were determined using DPV.

Similar principles of determination were used in 2018 when Liu *et al.* [125] described a sensitive voltammetric method for the determination of INDO in pharmaceutical preparations. The determination was based on a graphene-loaded nickel oxide nanoparticle (Gr-NiO) film obtained by one-step electrodeposition on a GCE. The authors used CV and DPV. As DPV showed a sharper peak at lower concentrations, it was used to determine INDO. The electrode showed a good storage stability, and there were no significant interferences; thus, the method is suitable for routine pharmaceutical analysis.

In the same year, Ragab *et al.* [126] developed DPV for simultaneous INDO and dantrolene determination in rat plasma. A much simpler HMDE was used as the working electrode, compared to previous research. This simple method showed high selectivity and sensitivity and could be applied in human plasma analyses.

In 2020, another successful voltammetric determination of INDO in plasma samples was described by Baezzat *et al.* [127]. They used a carbon ionic liquid electrode modified with TiO₂ nanoparticles as a working electrode. CV was used to determine the electrochemical parameters of INDO, and DPV was used for submicromolar quantification of the drug.

The last research on this topic was also published in 2020 when Petković *et al.* [128] developed a very sensitive, rapid, selective, and environmentally friendly method for the determination of mefenamic acid and INDO in pharmaceutical formulations. A special sensing platform, a boron-doped diamond electrode, was used to quantify the drugs without any time-consuming electrode modifications. The analytical procedure was based on the electrooxidation of the drug using DPV. This research showed that commercial electrode material can be successfully applied for the analysis of bioactive compounds and drugs.

The voltammetry is a very attractive method for various analyses, as well as for INDO determination. One of the most significant advantages of this method is the simple preparation of samples. In most of the studies previously described, INDO was dissolved in water. Few authors dissolved it in phosphate buffer, methanol, or acetonitrile. Real samples were usually deprotonated using acetonitrile or trichloroacetic acid followed by filtration and dissolving in appropriate buffer solution. Voltammetry was equally used for the analysis of biological samples and pharmaceutical preparations. In most cases, Ag/AgCl was used as a reference electrode and platinum wire as an auxiliary electrode. The working electrodes were modified with different nanomaterials to get the best analytical performance. The main disadvantage of the voltammetry is decreased selectivity, which can be improved by adjusting

solution conditions. It is the most commonly used electro-analytical method for INDO determination, which continued to develop until today.

3.11 Potentiometry

Potentiometry is an also electroanalytical method, but unlike amperometry, it measures potential in the system without drawing appreciable current. The potentiometric cell contains two electrodes: working and reference. Potentiometry is one of the simpler methods for INDO determination, although there are not many studies in this area. It is important to note that it is suitable for determining the release kinetics of INDO because it does not require complex sample preparation, and is sufficiently precise, selective, and fast for kinetic measurements. The sensors used in potentiometric measurements are ion-selective electrodes (ISEs), which selectively recognize the analyte. Their membrane is the most important part, which usually consists of an ionophore, as a sensing component, a plasticizer, and polyvinyl chloride (PVC). The components of the membrane and their proportions can be varied to develop an electrode with better properties (selectivity, sensitivity, LOD, working range, response time, accuracy, lifetime, etc.). Considering their advantages, such as simple preparation, low price, and short analysis time, these electrodes represent a successful alternative to standard methods. Attention should be paid to the solution conditions such as pH and ionic strength because it is known that they can affect the accuracy of the potentiometric determination.

The first ISE for INDO determination was developed in 1991 [129]. After a long pause, Aktaş and Ertokuş [130] continued with the potentiometric determination of INDO on a new level in 2008. The authors coupled classical potentiometric titrations with modern neural networks, using a glass-silver chloride electrode system, tetrabutylammonium hydroxide as a titrant, and an artificial neural network (ANN) as a multivariate calibration tool in the potentiometric titration. The ANN was used to model the complex nonlinear relationship among ibuprofen, INDO, and naproxen concentrations and the potential of the solutions measured after the addition of different volumes of the titrant. The optimized ANN could predict the concentrations of drugs in synthetic mixtures.

In 2009, Kormosh et al. [131] returned to the original INDO determination model by designing a potentiometric sensor with a polymeric membrane. They used the ion pair of INDO (anion) and rhodamine B (cation) as the

sensing material. The INDO was determined in pharmaceutical preparations using direct potentiometric measurements. The developed sensor was selective, fast, stable, and simple.

In 2012, Lenik and Wardak [132] described a similar way of determining INDO with a potentiometric sensor. The ion-exchange polymeric membrane of the ISE was based on an ion pair, tetraoctylammonium 1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetate. This research included the determination of basic sensor parameters and the determination of INDO in pharmaceutical preparation using direct potentiometric measurements. A lower LOD was obtained using this sensor, compared with other potentiometric sensors for INDO determination.

As like as voltammetry, potentiometric determination of INDO is characterized with simple preparation of samples (dissolving in water or acetonitrile, filtration, and addition of buffer solution). In all the studies described, INDO was determined in pharmaceutical samples. Although, few scientific studies describe potentiometry as an analytical method for INDO determination, it has great potential for further development and electrode modifications considering its significant advantages.

3.12 IMs

IMs are simple, fast, and sensitive methods based on specific antigen–antibody reaction. Extensive sample preparation or expensive instrumentation are not required for these methods, so they can be a good alternative to long-term chromatographic methods and can be used for routine pharmaceutical and clinical analyses. Two types of quantitative IMs are most commonly used to determine drugs, including INDO: enzyme-linked immunosorbent assay (ELISA) where antigens or antibodies are labeled using enzyme, and radioimmunoassay (RIA) where antigens or antibodies are labeled using radioisotope.

RIA was used for INDO determination in biological fluids in the 1970s [133,134].

After a long pause, scientists returned to IMs, but this time with a whole new approach. Huo et al. [135] applied a sensitive and specific indirect competitive ELISA method to determine the concentration of pharmaceutical INDO in different water samples. The sample preparation was simple and included filtration or filtration and dilution for surface water and wastewater, respectively. They covalently linked INDO to bovine serum albumin and ovalbumin by an anhydride ester method to prepare an immunogen and coating antigen. The authors immunized

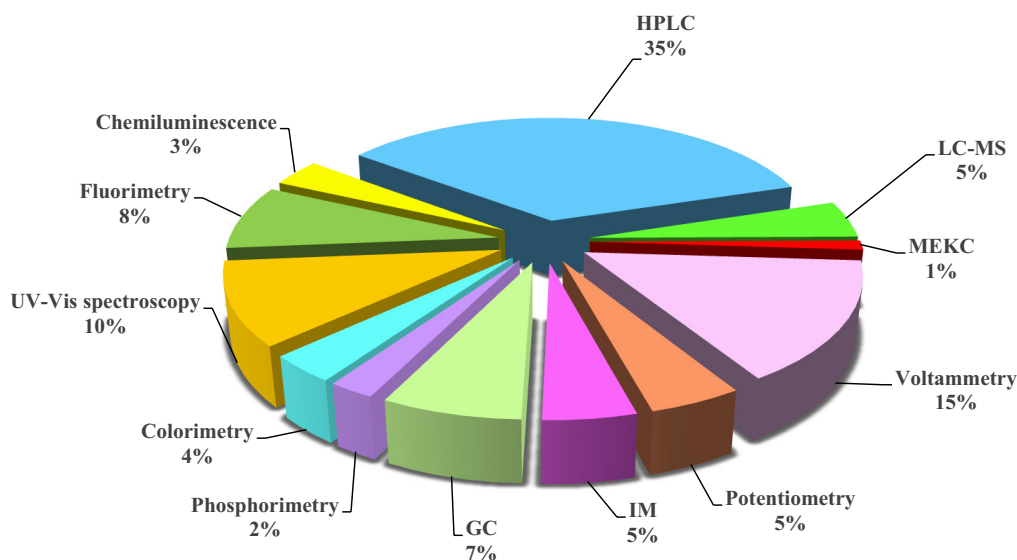


Figure 4: Distribution of analytical methods described in the literature for INDO determination in period between 1965 and 2020.

rabbits by standard immunization processes. The ELISA method began with the addition of antigen to the wells, after which samples and diluted antiserum were added. After incubation, substrate was added to develop color, and sulfuric acid to stop the enzymatic reaction. Measurements were performed spectrophotometrically. The results of the ELISA method were compared with those obtained using HPLC, with good correlation.

In 2011, IMs went a step further, when Skalka *et al.* [136] proposed an indirect competitive chemiluminescent ELISA method combined with an immunochemical sol-gel-based immunoaffinity purification (IAP) method, which was used for clean-up and concentration of INDO. IAP included application of serum to column doped with anti-INDO antiserum, elution with acetonitrile, evaporation under nitrogen, and dissolving in a mixture of phosphate buffer and methanol. The authors generated a polyclonal antibody for INDO and compared two microplate assays (ovalbumin and horseradish peroxidase formats) based on the ELISA method. This method, developed for INDO determination in human serum, can also be applied to monitor INDO concentrations in water samples.

Using RIA, picogram amounts of analyte can be measured considering the high specificity and sensitivity of this method. However, RIA includes handling and disposal of radioactive reagents, so in most of laboratories it is replaced by nonisotopic immunoassays such as ELISA. ELISA also is highly selective, sensitive, and efficient for the analysis of a large number of analytes. This sophisticated method has a few significant disadvantages, such as

reagent instability and high cost, which makes it less popular compared to electroanalytical methods.

Among the methods developed for INDO determination, in the period between 1965 and 2020, HPLC is the most frequently used (35% of the methods published in the literature) despite use of organic solvents and expensive instrumentation. Other commonly used methods are voltammetry (15% of published methods) and UV-Vis spectroscopy (10% of published methods), which is expected because these methods are simple, sensitive, easy to modify and optimize, and available in most laboratories. Although represented with 7% of all methods used for INDO determination, GC is rarely used today due to its drawbacks. Surprisingly, MEKC, as simple and environmentally friendly method, is the rarest method used, represented with only one research article (1% of all methods used for INDO determination). A graphical presentation of the percentage of methods used for INDO determination can be seen in Figure 4.

Different types of samples (biological and pharmaceutical) were analyzed with different analytical methods (Figure 5) due to the fact that complex systems, such as real biological samples, require more selective analytical methods. Voltammetry was used equally for both types of samples, thus confirming its good properties and wide application. Although UV-Vis spectroscopy is one of the most commonly used methods for INDO determination, all analyzed samples were pharmaceutical, indicating possible problems for INDO determination in complex matrices. However, chromatographic methods were mostly used to

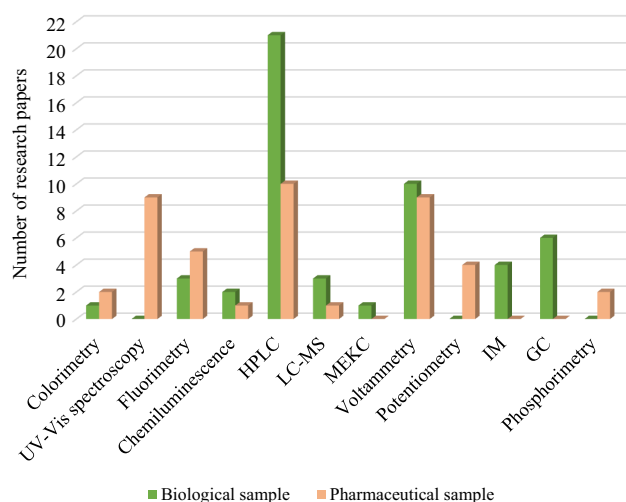


Figure 5: Distribution of analytical methods for INDO determination in relation to type of samples described in the literature in period between 1965 and 2020.

analyze biological samples due to good selectivity and specificity, which allows INDO determination in complex matrices. IMs were used only for INDO determination in biological samples due to the high specificity, and potentiometry was used only in pure pharmaceutical samples due to strong possible interferences of matrix compounds.

In addition, all methods presented in this review were checked if they are validated in accordance to the European Pharmacopoeia [18], British Pharmacopoeia [19], United States Pharmacopoeia [20], ICH guideline Bioanalytical method validation M10 [137], and Bioanalytical method validation guidance for industry [138]. Methods validated according to the ICH guideline Bioanalytical method validation M10 and Bioanalytical method validation guidance for industry are marked in Table 1. As the methods in pharmacopoeias are revised regularly, none of the methods described in this review have been validated according to current pharmacopoeias.

4 Conclusion

INDO is a widely used NSAID because of its analgesic, antipyretic, and anti-inflammatory properties. It is necessary to develop reliable, accurate, and efficient analytical methods for its determination to ensure the quality, safety, and efficiency of INDO drugs and to monitor INDO therapy. Also, it is important to monitor INDO and its metabolites in the environment.

This review describes the structural form of INDO, its structural modifications, physicochemical properties,

mechanism of action, pharmacokinetics, application, adverse effects, and drug interactions, with emphasis on analytical methods for the determination of INDO. Chromatographic methods are the most commonly used for INDO determination (almost 50% of all analytical methods used for INDO determination) due to good analytical performances despite the fact that these methods are not environmentally friendly, they generate toxic and carcinogenic waste, and commonly includes complicated sample preparation. However, modifications of mobile phase, used solvents, column length and particle size could lead to better results, so there is potential for future development.

Good alternative for chromatographic methods could be electrochemical techniques, especially because they do not require complicated sample preparation and expensive instrumentation. Voltammetry, as the second most commonly used method, proved to be appropriate for INDO determination, both in pure and real systems. Potentiometry was rarely used for INDO determination in spite of the fact that both methods could be easily improved using different types of electrodes, and functionalized nanomaterials for electrode modifications.

Optical methods were very popular for INDO determination, but lately, only UV-Vis spectroscopy has been used due to its simplicity. Great potential in optical methods development could be the introduction of nanomaterials with their unique properties and novel signal transduction approaches.

Among all the methods used for INDO determination, HPLC was the most sensitive technique with the lowest LOD ($1.07 \times 10^{-6} \mu\text{g}\cdot\text{mL}^{-1}$), but LC-MS and voltammetry also showed high sensitivity. According to all data collected and analyzed, HPLC, voltammetry and UV-Vis spectroscopy are methods that are still being developed, so in the future we can expect a further increase in sensitivity and even lower LOD.

This review presents 12 different analytical methods for INDO determination and 86 scientific investigations, including different strategies and their mostly successful applications. However, it is necessary to continue the research in this field to develop and validate new innovative analytical methods for INDO determination using green chemistry principles, as well as simple and fast sample preparation and analysis, which could be used for routine analysis.

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