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Development and validation of HPLC method for determination of indomethacin and its two degradation products in topical gel

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Abstract

Indomethacin forms by decomposition two degradation products: 4-chlorobenzoic acid and 5-methoxy-2-methylindoleacetic acid. They have to be monitored together with an active substance both during manufacturing process and storage of pharmaceuticals. European Pharmacopoeia (Ph. Eur. 4) describes titration method for determination of indomethacin, which is not very convenient in this case for practical use. Therefore, high performance liquid chromatography is the method-of-choice enabling determination of active substance and its degradation products during one-step procedure simultaneously and automatically.

We have developed a fast, simple and fully automated analytical method for determination of indomethacin and its two impurities in pharmaceutical preparation using HPLC with UV detection. Various stationary phases were tested, especially new types of Zorbax columns made by Agilent. While the conventional C18 stationary phases were not convenient enough to achieve quick and reliable separation, Zorbax-Phenyl analytical column (75 mm × 4.6 mm, 3.5 μm) enables separation of indomethacin and its two degradation products during 7.5 min. Chromatography was performed using isocratic elution with binary mobile phase composed of acetonitrile and 0.2% phosphoric acid (50:50, v/v) at flow rate 0.6 ml/min. Even faster separation of standards was obtained with analytical column Zorbax SB-CN (150 mm × 4.6 mm, 5 μm). The separation was effected with mobile phase of the same composition, only the flow rate was increased to 1.2 ml/min. The analytical run was shortened to 5 min. Both methods use detection wavelength 237 nm and both can use either ketoprofen or flurbiprofen as internal standard for quantitation.

The first method was finally chosen for validation because of the occurrence of placebo interferences in the case of using Zorbax SB-CN. System suitability parameters and validation parameters including method precision, accuracy, linearity, selectivity and robustness were set up. Afterwards, the method was successfully applied for the practical determination of indomethacin and its degradation products in a topical gel and for compound degradation control during stability studies.
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Keywords: Indomethacin; HPLC; Pharmaceuticals; Degradation products

1. Introduction

Indomethacin – chemically 1-(4-chlorbenzoyl)-5-methoxy-2-methylindoleacetic acid – is a non-steroidal anti-inflammatory (NSAID), analgetic and antipyretic drug. Its effect is based on inhibition of cyclo-oxygenase (COX). It is frequently used for the treatment of symptoms of rheumatoid arthritis. In veterinary medicine, it is effective in treatment of inflammatory processes related to infectious diseases. The drug is usually administered orally. It can also be administered intravenously or as a suppository and topical gel [1].

By decomposition it forms two degradation products: 4-chloro-benzoic acid and 5-methoxy-2-methylindoleacetic acid. They have to be monitored together with an active substance both during manufacturing process and storage of pharmaceuticals with aim to control the quality (undesirable

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impurities or level of degradation products) and quantity (active substance assay) of the pharmaceutical product.

The European Pharmacopoeia (Ph. Eur. 4) [2] uses a titration method for the determination of indomethacin. The substance is titrated with 0.1 M sodium hydroxide and blank titration had to be carried out simultaneously. This is time consuming and impractical for routine analyses of pharmaceutical samples, especially during stability studies and quality control in the manufacturing process, where there could be many samples to be controlled, often in replicates.

The USP 26 method for indomethacin substance determination [3] recommends using of (4 mm × 30 mm, 5 μm) analytical column containing L1 packing (ODS chemically bonded to silica). The amount of indomethacin active substance in capsules and in suppositories is determined using absorption spectrophotometry. All these methods determine the amount of indomethacin only, none of them includes degradation product determination. This is stated in three other monographs—in indomethacin oral suspension, in indomethacin for injection and in indomethacin extended-release capsules. In the first two monographs HPLC on reversed phase is used for determination of indomethacin active substance and 4-chlorobenzoic acid as an impurity in one analytical run. In the monograph extended release capsules, indomethacin active substance is determined by absorption spectrophotometry 4-chlorobenzoic acid assay determination is performed by RP-HPLC.

As it can be clearly seen, none of these methods is developed and validated for determination of indomethacin and both its impurities in the presence of internal standard. There are also many other methods published in scientific journals as well. The oldest methods for indomethacin assay determination are even from the 1980s of last century. They usually include indomethacin, eventually another anti-inflammatory drugs determination in various biological fluids by means of RP-HPLC.

Recently developed methods for indomethacin determination use modern techniques. Löffler and Ternes employ LC-APCI-MS-MS [4] for determination of acidic compounds including indomethacin (the others, eg. diclofenac, fenoprofen, ibuprofen and gemfibrozil) in the negative mode of ionisation. For all pharmaceuticals, analytical column used was LiChrospher RP-18 (125 mm × 3 mm, 5 μm) with limits of quantitation for all acidic compounds ranged from 0.4 to 8 ng/g.

Acidic drugs (indomethacin, ibuprofen, diclofenac, bezafibrate and others) were also analysed by Miao et al. [5]. They used liquid chromatography–electrospray ionisation tandem mass spectrometry. Analyses were carried out using Genesis C_{18} column (150 mm × 2.1 mm, i.d., 4 μm). The method gave 58.5% recovery for indomethacin and limits of detection (LOD) for this compound was 10 ng/l.

Abed-Hamid et al. [6] developed method for the determination of diclofenac sodium, flufenamic acid, indomethacin and ketoprofen by LC-APCI-MS. This method gives mean recovery percentages of 99.5–101.5%. LOD for indomethacin was 4.0 ng/ml and limits of quantitation (LOQ) was 100 ng/ml.

All these methods have very good sensitivity and selectivity for stated compounds. Their main disadvantage is instrument complexity and the high cost of analysis. There are many other methods using simple HPLC with ultraviolet or fluorimetric detection. They usually determine indomethacin together with another non-steroidal drugs, steroidal anti-inflammatory drugs in biological fluids, or they deal with distribution study [7], and the determination of indomethacin residuals in poultry [8].

Indomethacin together with hydrocortizone, dexamethazone, phenylbutazone and oxyphenbutazone in equine serum was determined by Grippa et al. [9]. The method uses simple HPLC with UV detection at 254 nm on C18 stationary phase. The limit of quantitation for indomethacin was 0.5 μg/ml. Indomethacin and other NSAIDS are often determined in human urine [10–14], human serum [15,16], aqueous humor [17] and most commonly in human plasma [12,18–21] using HPLC methods.

There are only two methods for determining substances derived from indomethacin. Vree et al. [12] determined indomethacin and its metabolites (*O*-desmethylindomethacin, deschlorobenzoyl indomethacin) and their glucuronides in human plasma and urine. Smith and Benet [14] determined indomethacin together with its two primary metabolites (demethylmetabolite and dechlorbenzoylmetabolite) in urine. It is important to emphasize that in spite of the large number of HPLC methods for indomethacin determination, there is no method for determining indomethacin and its two degradation products in one analysis simultaneously including internal standard as well. To achieve this aim there is a need to develop a new, simple and fast analytical method for the simultaneous determination of these substances in pharmaceutical formulations. In our study, high performance liquid chromatography with UV detection was chosen for separation, identification and quantitation of indomethacin active substance and its two degradation products 4-chlorobenzoic acid and 5-methoxy-2-methylindoleacetic acid (Fig. 1) in the topical pharmaceutical formulation indomethacin and indobene gel.

2. Experimental

2.1. Chemicals and reagents

Working standards of indomethacin, 4-chlorobenzoic acid, 5-methoxy-2-methylindoleacetic acid, flurbiprofen and ketoprofen (tested internal standards), were used for the purpose of this study. Indomethacin active substance (1-(4-chlorbenzoyl)-5-methoxy-2-methylindoleacetic acid) was provided by Herbacos (Pardubice, Czech Republic), as was the Ketoprofen (2-(3-benzoylphenyl) propionic acid) used as an internal standard. Flurbiprofen (2-fluoro-α-methyl-

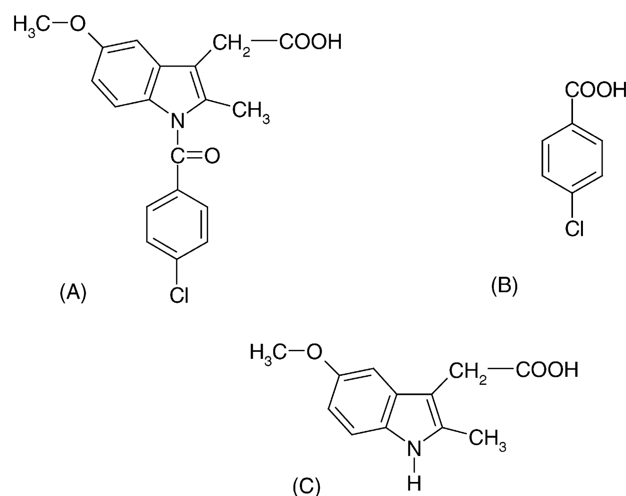

Fig. 1. Molecular structures of active substance and degradation products in indomethacin gel: (A) indomethacin, (B) 4-chlorobenzoic acid, (C) 5-methoxy-2-methylindoleacetic acid.

biphenylacetic acid) was obtained from Sigma-Aldrich (Prague, Czech Republic).

Both impurities: 4-chlorobenzoic acid and 5-methoxy-2-methylindoleacetic acid were provided by Sigma-Aldrich (Prague, Czech Republic). All these compounds were checked against European Pharmacopoeia CRS standards (Strasbourg, France).

Phosphoric acid 85% p.a. was obtained from Merck (Darmstadt, Germany). Acetonitrile, Supra-gradient was obtained from Biotech (Scherlau Chemie, Germany). HPLC grade methanol was provided by Sigma-Aldrich (Prague, Czech Republic).

HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and it meets European Pharmacopoeia requirements.

2.2. UV spectra measurement

UV spectra of tested compounds were measured by spectrophotometer Hewlett Packard 8453 equipped with software Chemstation (UV–vis for biochemistry).

Methanol solutions of individual compounds were prepared so that the concentration was similar to the expected value in pharmaceutical preparation. The final concentrations were approximately: indomethacin (250.0 mg/l), 4-chlorobenzoic acid (5.0 mg/l), 5-methoxy-2-methylindoleacetic acid (5.0 mg/l), ketoprofen (10.0 mg/l) and flurbiprofen (10.0 mg/l).

2.3. Chromatography

A Shimadzu LC-2010 C system (Shimadzu, Japan) was used to perform all of the analyses. Detection of individual compounds was done with built-in UV–vis detector. The instrument is also equipped with column oven enabling temperature control. The built-in auto-sampler was conditioned at 25 °C. Chromatographic software Class VP 5 was used for data collection and processing.

Various analytical columns were tested. Stationary phases based on octadecylsilca sorbent Supelco Discovery™ C18 (125 mm × 4.0 mm, 5 μm) purchased from Sigma-Aldrich (Prague, Czech Republic) and Zorbax SB C18 (50 mm × 4.6 mm, 1.8 μm) were not successful in analytical separation, while Zorbax SB-Phenyl (75 mm × 4.6mm, 3.5 μm) and Zorbax SB-CN (150 mm × 4.6 mm, 5 μm) gave good results. All Zorbax columns were obtained from Agilent Technologies (Prague, Czech Republic).

2.4. Reference standard preparation

Firstly, the stock solutions of internal standard and impurities were prepared. The stock solution of internal standard was prepared by dissolving 50 mg of ketoprofen working standard in 100 ml of methanol. The stock solutions of impurities were prepared by dissolving 5.0 mg 4-chlorobenzoic acid and 5-methoxy-2-methylindoleacetic acid, respectively, in 100 ml of methanol. Reference standard solution for indomethacin gel analysis was prepared in 100 ml volumetric flask by dissolving of 25.0 mg of indomethacin in methanol. Afterwards, 2.0 ml of internal standard ketoprofen stock solution and 10.0 ml of each impurity stock solution were added before the flask was made up to the volume with methanol.

Working solutions of internal standards were prepared by diluting 10.0 ml of the internal standard stock solution in methanol to a volume of 500 ml. Thus, the final concentration of internal standard ketoprofen was always approximately 10 mg/l. It was necessary to keep stock solutions and standard solutions at a reduced temperature (4 °C) for stability reasons.

2.5. Sample preparation

0.5 g of topical indomethacin gel (which corresponds to 5.0 mg of indomethacin active substance) was accurately weighed and was transferred into 50.0 ml centrifuge tube. Twenty millilitres of internal standard working solution in methanol (1 mg/100 ml of ketoprofen in methanol) were added. This mixture was sonicated for 10 min and then centrifuged for 15 min at 1300 g (laboratory centrifuge EBA 21, Hettich, Tutlingen, Germany). The supernatant was injected directly into the chromatographic system (or it was filtered through the 0.45 μm filters if it was necessary. Sometimes it could happen that the supernatant is not sufficiently clear).

2.6. Method validation

The objective of method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines Q2A and Q2B [22]. Recommend validation characteristics depend on the type of analytical procedure. An important part of method validation is system suitability test (SST), details of which are usually given in pharma-

copoeias. We have established a number of theoretical plates, peak asymmetry, resolution of individual compounds and repeatability of injection (retentions times and peak areas were checked).

Method validation characteristics were tested in accordance with ICH guidelines and pharmacopoeial requirements. We have established method accuracy (% recovery and % R.S.D. of individual measurements) and method precision (% R.S.D.) using six samples in three replicates. Linearity (correlation coefficient) was tested in the range 20–150% for active substance indomethacin (100–500 mg/l). Degradation products were tested in a range 0.1–5% of active substance content (0.25–12.5 mg/l). Six concentration levels were used. Method selectivity was verified by comparison of sample of pharmaceutical preparation, standard solution and placebo chromatograms. Limits of detection and quantitation were provided for two degradation products 4-chlorobenzoic acid and 5-methoxy-2-methylindoleacetic acid. Calculation was made by means of signal-to-noise ratio method. Standard solution short-term stability was tested at ambient temperature (25 °C) and at 4 °C. Also, method robustness with regard to mobile-phase composition was studied.

3. Results and discussion

3.1. Chromatographic conditions

The European Pharmacopoeia uses a titration method for indomethacin assay, while the analytical methods described in USP 26 [3] use many methods for indomethacin determination. They include spectrophotometric assay determination and various HPLC methods. Some of them are able to determine 4-chlorobenzoic acid as an impurity as well, but none of the methods enables determination of quantity of 5-methoxy-2-methylindoleacetic acid simultaneously with indomethacin and 4-chlorobenzoic acid.

HPLC with UV detection was chosen as a simple, fast and effective separation method for determination of indomethacin and its two degradation products. The absorption wavelength for detection of the compounds was chosen especially with regard to absorption spectra of the degradation products. Both degradation products give a lower detector response compared to indomethacin active substance and, of course, they are present in much lower quantities than indomethacin. The results from the UV spectra of individual compounds can be seen in Table 1.

Many analytical columns were tested in the development of this method. Firstly, a conventional stationary phase based on octadecylsilica sorbent Supelco Discovery™ C18 (125 mm × 4 mm, 5 μm) was tested. Mobile phases consisting of water, methanol, acetonitrile and 0.2% phosphoric acid in different rates and combinations were applied, but the results still were not satisfactory. The main problem was placebo interferences at retention times of compounds, which should be detected. This problem was not solved using a Zorbax SB C18 (50 mm × 4.6 mm, 1.8 μm) analytical column as well. All tested compounds were well separated, but we were not able to eliminate the placebo interference with 4-chlorobenzoic acid.

Table 1
UV spectra data from individual compounds

Tested compound	Maximum 1 (nm)	Maximum 2 (nm)	Maximum 3 (nm)
Indomethacin	224	230	304
4-Chlorobenzoic acid	237	328	356
5-Methoxy-2-methylindoleacetic acid	201	224	253
Flurbiprofen (internal standard 1)	206	221	246
Ketoprofen (internal standard 2)	202	255	–

Testing different stationary phases was more successful. Other analytical columns from the Agilent Zorbax group of analytical columns were tested. Again, mobile phases of similar compositions were tested. The best results were obtained using a mobile phase consisting of acetonitrile and 0.2% phosphoric acid in different ratios and mobile-phase flow rates. Two more different stationary phases Zorbax SB-Phenyl (75 mm × 4.6 mm, 3.5 μm) and Zorbax SB-CN (150 mm × 4.6 mm, 5 μm) were tested.

Analytical column Zorbax SB-Phenyl (75 mm × 4.6 mm, 3.5 μm) enables separation of indomethacin and its two degradation products during 7.5 min. Chromatography was performed using isocratic elution with binary mobile phase composed of acetonitrile and 0.2% phosphoric acid (50:50, v/v) at flow rate 0.6 ml/min.

Even faster separation of standards was obtained with analytical column Zorbax SB-CN (150 mm × 4.6 mm, 5 μm). The separation was effected with mobile phase of the same composition, only the flow rate was increased to 1.2 ml/min. The analytical run was shortened to 5 min. Both methods use detection wavelength 237 nm and both can use either ketoprofen or flurbiprofen as internal standard for quantitation. In the case of the analysis of ointments and gels, the sample preparation procedure requires the use of the method of internal standard due to the common problems related with method recovery. All analyses were kept at ambient temperature.

The first method using Zorbax SB-Phenyl column was finally chosen for validation because the placebo interferences in this case were eliminated. Separation of all compounds at these conditions takes about 7.5 min as it can be seen in Fig. 2.

System suitability parameters and validation parameters including method precision, accuracy, linearity, selectivity and robustness were set up. Afterwards, the method was successfully applied for the practical determination of indomethacin and its degradation products in topical gel and for the control of degradation compounds during stability studies.

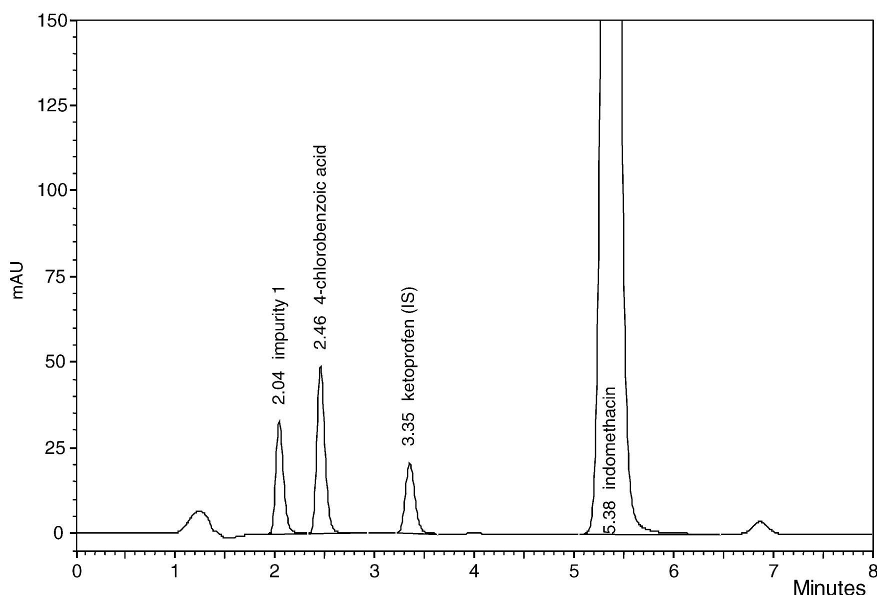

Fig. 2. Chromatogram 1: LC–UV chromatogram (237 nm) obtained from the analysis of compounds in standard solutions, indomethacin (250.0 mg/l), ketoprofen (10.0 internal standard milligram per litre), 4-chlorobenzoic acid (5.0 mg/l) and 5-methoxy-2-methylindoleacetic acid (5.0 mg/l) = impurity 1.

3.2. Isolation procedure

The isolation procedure was developed on the basis of methods for analysis of topical preparations routinely used in our laboratory. Two extraction media were tested—acetonitrile and methanol. Finally, methanol was chosen, because there was lower placebo background using this extraction agent. Internal standard ketoprofen (10 mg/l) was added. The procedure, as described above, gives recovery from 95.05–100.04% (these include individual results for all compounds, while the validation table results includes the mean percentage of recovery). The chromatogram shown in Fig. 3 is an illustration of the separation of all compounds tested after isolation from a pharmaceutical preparation. There are no other substances, which may coelute with tested analytes as was verified during method validation–selectivity testing.

3.3. Validation of methods

After the method development and optimisation is finished, it is necessary to accomplish method validation. Fol-

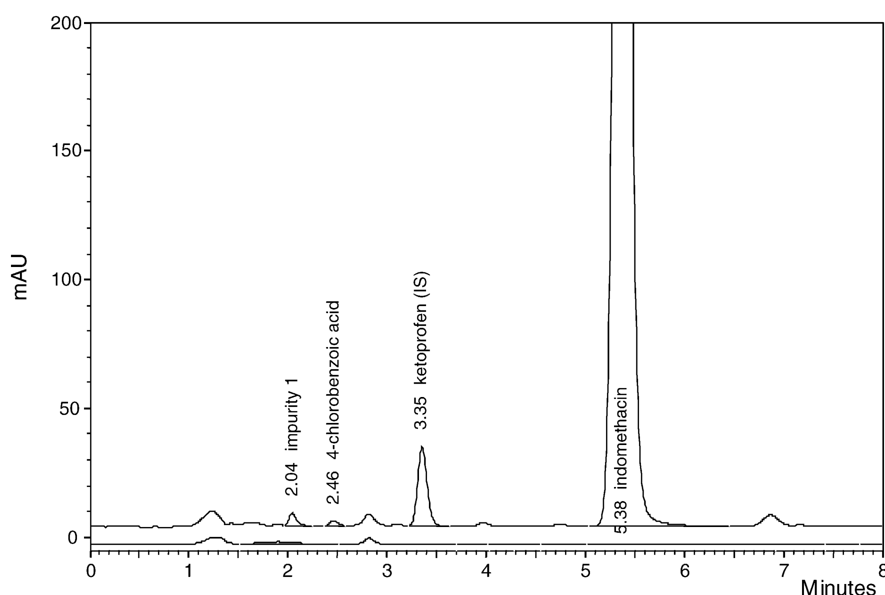

Fig. 3. Chromatogram 2: LC–UV chromatogram (237 nm) obtained from the analysis of compounds in pharmaceutical formulation indomethacin gel (with placebo chromatogram to demonstrate method selectivity) 5-methoxy-2-methylindoleacetic acid = impurity 1.

Table 2
Short-term stability study for tested compounds

Time of storage (h)	Concentration changes during storage (%)					
	Indomethacin		4-Cholrobenzoic acid		5-Methoxy-2-methylindoleacetic acid	
	25 (°C)	4 (°C)	25 (°C)	4 (°C)	25 (°C)	4 (°C)
0	100.00	100.00	100.00	100.00	100.00	100.00
24	+0.72	+0.49	+0.03	+0.18	−1.10	−0.24
48	+0.87	+0.86	+0.24	+0.82	−3.03	+0.21
72	+0.72	+0.89	−0.25	+0.76	−3.71	+0.12

Table 3
The influence of small changes in mobile-phase composition (method robustness)

Mobile-phase composition	Indomethacin		4-Chlorobenzoic acid		5-Methoxy-2-methylindoleacetic acid	
	t_R	A	t_R	A	t_R	A
ACN:acid (40:60)	16.08	98.17	3.80	99.92	2.87	97.91
ACN:acid (45:55)	8.41	98.53	2.93	99.57	2.33	100.34
ACN:acid (50:50)	5.40	100.00	2.46	100.00	2.04	100.00
ACN:acid (55:45)	4.18	99.55	2.23	101.22	1.90	101.02
ACN:acid (60:40)	3.15	99.90	1.98	100.49	1.74	93.54

ACN: acetonitrile; acid: 0.2% phosphoric acid; t_R: retention time; A: peak area (%).

lowing the international ICH guidelines for analytical method validation (Q2A and Q2B) together with in-house rules of our laboratory, both SST and validation parameters were measured.

Recommended validation characteristics including method precision (%R.S.D.), method accuracy (% of recovery, %R.S.D.) linear range (correlation coefficient) and method selectivity using placebo of pharmaceutical formulation were set up. LOD and LOQ were provided for degradation products 4-chlorobenzoic acid and 5-methoxy-2-methylindoleacetic acid by means of the method of signal-to-noise ratio.

The short-term stability of the compounds of interest was tested. Standard solutions were kept at ambient temperature (25 °C) and at decreased temperature (4 °C). Changes in content of 5-methoxy-2-methylindoleacetic acid, higher than 1%, were observed during the storage at ambient temperature (Table 2). The same changes were not observed during storage at decreased temperature, all compounds showing peak area changes up to 1%. This indicates that stock solutions and standard solution should be stored at 4 °C.

Influences of small changes in mobile-phase composition (±10%) were studied to determine robustness of the method. Peak areas and retention time changes were observed. Results are clearly seen in Table 3. Peak area values were influenced less, up to 2% for indomethacin and 4-chlrobenzoic acid. Concerning 5-methoxy-2-methylindoleacetic acid, the changes were more significant, especially with a 10% change in mobile-phase composition. The retention times of the compounds of interest were influenced significantly only in the

Table 4
Method validation results for individual compounds

	Indomethacin	4-Chlorobenzoic acid	5-Methoxy-2-methylindoleacetic acid	Limits
SST				
Theoretical plates[a]	7655	4000	3103	$N > 1500$
Asymmetry[a]	1.06	1.15	1.24	$T < 1.5$
Resolution[a]	5.59	2.70	3.11	$R_{ij} > 1.5$
Repeatability-t_r[b]	0.34	0.23	0.18	R.S.D. < 1%
Repeatability-A[b]	0.18	0.66	0.45	R.S.D. < 1%
Validation				
Precision[c] (%R.S.D.)	1.39	3.25	3.10	R.S.D. < 5%
Linearity (corelation coefficient)	0.9999[d]	0.9999[e]	0.9999[e]	$R > 0.9990$
Accuracy[c] (%R.S.D.)	0.70	0.62	1.13	R.S.D. < 5%
Accuracy[c] (%recovery)	99.53	98.27	96.22	100 ± 5%
Selectivity	No interference	No interference	No interference	
LOD (mg/ml)	–	5.69×10^{-5}	2.25×10^{-4}	–
LOQ (mg/ml)	–	1.90×10^{-4}	7.5×10^{-4}	–

[a] Made in three replicates.
[b] Made in six replicates.
[c] Six samples injected three times each.
[d] At 20, 50, 80, 100, 120, 150% levels, three replicates.
[e] At 0.1–5.0% range of active substance concentration.

case of indomethacin. Despite these changes in retention time the separation was sufficient, thus the quantitation was still possible.

All validation results are summarized in Table 4, showing that they are in agreement with the expected values.

3.4. Pharmaceutical applications

The novel analytical method for the determination of the active substance indomethacin and its two impurities 4-chlorobenzoic acid and 5-methoxy-2-methylindoleacetic acid as developed by this work is effective, fast and meets all criteria for method validation. The method can be successfully applied for practical use. It is used for indomethacin assay determination in the pharmaceutical preparation indomethacin gel and it was also applied for analysis of indobene gel. For this purpose, the method has not been not validated so far, but it could be simply used as it seems from the preliminary analyses. The method also enables degradation process control in stability studies of these formulations, because the limits of detection and quantitation for degradation products were provided

4. Conclusion

We have developed a fast, simple and fully automated analytical method for simultaneous determination of indomethacin and its two impurities in pharmaceutical preparation using HPLC with UV detection. The method uses the internal standard ketoprofen for quantitation and elimination of deviations during isolation procedure. An analytical run takes less than 7.5 min.

Separation of compounds is very fast, with sufficient resolution, good reproducibility and peak asymmetry. Validation of this method was accomplished, getting results meeting all requirements. The method is simple, reproducible, with a good accuracy and precision. It allows the analysis of all compounds of interest in the topical gel indomethacin simultaneously in one analytical run during a short period of time. After supplementary validation it is supposed to be used as well for the other pharmaceutical formulation indobene gel.

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