Introduction

Indomethacin: a widely used non-steroid anti-inflammatory agent

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) commonly used as a prescription medication to reduce fever, pain, stiffness, and swelling. It works by inhibiting the production of prostaglandins, molecules known to cause these symptoms [1]. Indomethacin has also been used clinically to delay premature labor [2], reduce amniotic fluid in polyhydramnios [3, 4]. Indomethacin is also used in closure of the ductus arteriosus in newborn humans [5]. Indomethacin is a potent drug with many serious side effects and should not be considered an analgesic for minor aches and pains or fever. The medication is better described as an anti-inflammatory, rather than an analgesic. Indomethacin can also affect warfarin and subsequently raise INR.

Some conflicting data in its studies have led to a reexamination of the substance’s stability. It has been considered, that the lack of indomethacin activity in some biological studies may have resulted from injection of inactive solutions. [6].

As described in literature indomethacin forms by decomposition two degradation products: 5-methoxy-2-methylindol-3-yl acetate (A) and 4-chlorobenzoic acid (B) (Fig. 1) [7, 8]. The latter has to be monitored together with an active substance both during manufacturing process and storage of pharmaceuticals, due to some evidences of its hepatotoxicity [9].

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EVALUATION OF THE STABILITY OF INDOMETHACIN SUBSTANCE UNDER A MODEL OF PHYSIOLOGICAL CONDITIONS, USING MODIFIED AND VALIDATED RP-HPLC METHOD

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Abstract. A fast, simple and fully automated RP-HPLC analytical method with UV detection for determination of indomethacin substance and its degradation product 4-chlorobenzoic acid was modified and validated. The analysis was performed at isocratic conditions, applying a mobile phase of acetonitrile and 0.5% orthophosphoric acid (50:50, v/v) at flow rate 1.5 ml/min. The system’s suitability parameters and validation parameters were set up. The modified and validated method was successfully applied for determination of stability of indomethacin substance under a model of physiological conditions. The analyzed indomethacin substance was established to be stable in acid and neutral media, while a hydrolysis occurs in alkali media. A first order rate constant for the degradation of indomethacin in alkali media was determined.

Key words: stability, indomethacin, RP-HPLC, validation

Fig. 1. Chemical structure of the degradation products of Indomethacin.
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 [10].

The aim of our study is to modify and validate an automated RP-HPLC method for simultaneous identification and quantitation of indomethacin substance and its degradation product 4-chlorobenzoic acid.

**Materials and methods**

**Reagents**

LC-grade methanol and acetonitrile were supplied from Merck (Germany). All other chemicals and reagents were of analytical grade. HPLC grade water was purified using a Milli-Ro®-15 Water Purification System (Millipore, Bedford, MA, USA), which consists of a Super-C® carbon cartridge, two Ion-X® ion-exchange cartridges and an Organex-Q® cartridge. The water was filtered through a 0.22μm Millipak® stack filter prior to use.

Indomethacin RS and 4-chlorobenzoic acid RS were used as standards.

**HPLC system**

A modular HPLC system LC-10A Shimadzu (Japan) arranged with a LC-10A pump, solvent degasser DGU-3A, Rheodyne injector, column oven CTO-10A, SPD-M10A fixed wavelength detector and communication bus module CBM-10A was used. A Luna C18 (2), 250 mm x 4.6 mm, 5 μm column was applied as stationary phase. The analysis was carried out at an ambient temperature with injection volume of 20 μl. The UV detector was set at 240 nm.

**Selection of a mobile phase.**

As a result from the performed analysis a mobile phase consisting of 50 volumes 0.5 % v/v orthophosphoric acid and 50 volumes of acetonitrile was used in the current assay with a flow rate of 1.5 ml/min.

**Preparation of buffers.**

All buffer solutions were prepared according to the requirements of European Pharmacopoeia [11].

**Preparation of the standard solutions.**

Reference stock solutions of 4-chlorobenzoic acid (0.1 mg/ml) and indomethacin (0.5 mg/ml) were prepared in acetonitrile and filtered through 0.45-μm membrane filter. Calibration solutions for indomethacin were prepared by diluting the reference stock solution to furnish concentrations in the range 25.00-200.0 μg/ml. Calibration solutions for 4-chlorobenzoic acid were prepared by diluting the reference stock solution to obtain concentrations in the range 5.00-40.00 μg/ml. The mobile phase was applied as solvent for dilutions. 20 μl of these solutions were injected in triplicate into the HPLC system and the peak areas were recorded.

**Preparation of test solutions.**

In the kinetic run, the reaction was initiated by dissolving 0.001 g of the analyzed indomethacin substance to 10.00 ml of preheated corresponding buffer solution pH 2.0; 1mM (Solution 1), pH 7.4; 1mM (Solution 2) and pH 9.0; 1mM (Solution 3) obtaining a final concentrations of 100 μg/ml.

**Stability evaluation**

The obtained solutions were maintained at 37±0.2°C for a total time of 120 min. Aliquot samples of 20 μl from the analyzed solutions were taken at definite time intervals (0, 30, 60, 90 and 120 min.) and the corresponding chromatograms were recorded. The progress of hydrolysis was monitored by means of the modified chromatograms.

The objective of the proposed study is to modify and validate a stability indicating HPLC method for simultaneous estimation of indomethacin substance and its hydrolytic product 4-chlorobenzoic acid.

**Results and discussion**

**Validation and chromatographic procedure**

Validation of an analytical procedure is performed in order to demonstrate that the procedure is suitable for its intended use. [12-14]. This process is essential as it serves to ensure that the quality of analytical data generated is both reliable and accurate, and is also capable to identify potential problems with the method [14-16].

In this assay the following eight parameters, outlined by the USP and ICH [12, 17] for evaluation of an analytical procedure, like accuracy, precision, specificity, limit of detection (LOD) and limit of quantitation (LOQ), linearity and range were determined. These parameters are specific to the validation of a developed method, and forms a part of an overall validation process, which includes validation of hardware and software used and verification of system suitability and performance [17].

In the presented work the selection of the mobile phase was based on methods published in the literature [18] and on the literary methods for the individual analysis of indomethacin and 4-chlorobenzoic acid [8, 19, 20]. The applied modification was initi-
ated from the necessity to develop simple and fast analytical method for simultaneous determination of indomethacin and its possible hydrolytic product 4-chlorobenzoic acid.

For improvement of the chromatographic behavior the influence of buffer molarity and pH were assessed and considered. For the assessment of the effect of these variables, the analyzed compounds were detected at the defined wavelength and 0.1 AUFS at 1.5 ml/minute [21].

Results from validation procedure.

The proposed method was validated with respect to specificity, selectivity, linearity, precision, accuracy, limit of quantitation (LOQ) and limit of detection (LOD).

Specificity

Specificity is defined as the ability to measure the analyte in the presence of other components that may be expected in the sample matrix, accurately and specifically [12, 13, 17, 22, 23]. Specificity was assessed by comparing chromatograms obtained from analysis of a placebo solution and reference solutions. No other peaks were observed at the retention times of indomethacin and its degradation product 4-chlorobenzoic acid, indicating that interfering substances were not present.

Selectivity

Under the proposed chromatographic conditions the obtained retention times for standard solution containing a mixture of the tested 4-chlorobenzoic acid RS and indomethacin RS were 2.074 and 9.857 min., respectively. From the chromatogram shown in Fig. 2, it is evident, that the analyzed compounds were completely separated from each other (Rs = 7.783), which indicated that the method is selective and could be used for their simultaneous identification, quantification and in purity tests.

Linearity

The linearity of the range of detectability is dependent on Beer’s Law, such that the absorbance of a solute is directly proportional to its concentration in the solution [24]. Linearity in this range is dependent on both the compound analyzed and the detector used [22].

In our study the linearity of the method was determined at eight concentration levels ranging from 25.00 to 200.0 μg/ml for indomethacin and 5.00 to 40.00 μg/ml for 4-chlorobenzoic acid. The calibration curves were constructed by plotting peak areas versus concentrations of the analyzed compounds, and the regression equations were calculated. Each response was the average of three determinations. The obtained calibration curves for each of the analyzed compounds are presented on Fig. 3.

![Fig. 2. Cromatogram of standard solution, containing 4-chlorobenzoic acid RS and indomethacin RS.](image)

![Fig. 3. Calibration curves for linearity determination for Indomethacin (a) and 4-chlorobenzoic acid (b).](image)
An $r^2$ value of >0.990 was considered to be sufficient to demonstrate linearity of the method. The calibration curve was linear over the concentration range studied, with $r^2 = 0.9988$ for indomethacin and $r^2 = 0.9915$ for 4-chlorobenzoic acid. The equations of the regression lines were $y = 3.8949x - 4.5002$ and $y = 19.413x - 23.129$ for indomethacin and 4-chlorobenzoic acid, respectively.

**Precision**

According to ICH precision is usually expressed as the percentage relative standard deviation (%RSD) [12, 13, 17].

The precision of the proposed analytical system was investigated by performing six consecutive replicate injections of the same standard solution. The standard deviation (SD) and relative standard deviation (%RSD) obtained are listed in Table 1.

The low %RSD values indicated that the method is precise.

**Limit of quantitation and limit of detection**

The limits of quantitation and limits of detection were calculated from the standard deviation of responses and slopes using signal-to-noise ratio. The quantitation limits for Indomethacin and 4-chlorobenzoic acid were 0.3 μg/ml and 0.9 μg/ml, respectively. The calculated detection limits were 0.06 μg/ml and 0.3 μg/ml, respectively for the analyzed indomethacin and 4-chlorobenzoic acid.

The assessed system suitability parameters and validation parameters determined that the method may be successfully applied for practical determination of indomethacin and its degradation product 4-chlorobenzoic acid for following and control of degradation of the active substance during stability studies.

**Results from stability evaluation of indomethacin RS.**

Three solutions were prepared according to the above mentioned procedure:

**Solution 1** with hydrochloric acid buffer giving a pH value of 1.2 – close to the physiological pH in the stomach;

**Solution 2** with phosphate buffer giving a pH value of 7.4 – equal to the physiological pH in the blood plasma and

**Solution 3** with solvent citrate buffer giving a pH value of 9.0 – close to the physiological pH in the small intestines.

The solutions were kept at 37°C and aliquot samples of 20 μl were taken at definite time intervals (0, 30, 60, 90 and 120 min.). The drawn aliquots were analyzed with the modified HPLC method and the obtained chromatograms were recorded. The corresponding peak areas are presented in Table 2, Table 3 and Table 4 for Solution 1, Solution 2 and Solution 3 respectively.

### Table 1. Precision of the method

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (μg/ml)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>100.05</td>
</tr>
<tr>
<td>4-chlorobenzoic acid</td>
<td>19.89</td>
</tr>
</tbody>
</table>

### Table 2. Determined peak area for indomethacin and 4-chlorobenzoic acid for Solution 1 for the indicated time intervals.

<table>
<thead>
<tr>
<th>Number</th>
<th>Time (min)</th>
<th>Rt (min) indomethacin</th>
<th>Peak area of indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0</td>
<td>8.952</td>
<td>1090624</td>
</tr>
<tr>
<td>2.</td>
<td>30</td>
<td>8.954</td>
<td>1142932</td>
</tr>
<tr>
<td>3.</td>
<td>60</td>
<td>8.789</td>
<td>1129844</td>
</tr>
<tr>
<td>4.</td>
<td>90</td>
<td>8.775</td>
<td>1025257</td>
</tr>
<tr>
<td>5.</td>
<td>120</td>
<td>7.898</td>
<td>1151147</td>
</tr>
</tbody>
</table>

### Table 3. Determined peak area for indomethacin and 4-chlorobenzoic acid for Solution 2 for the indicated time intervals.

<table>
<thead>
<tr>
<th>Number</th>
<th>Time (min)</th>
<th>Rt (min) indomethacin</th>
<th>Peak area of indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0</td>
<td>5.208</td>
<td>578048</td>
</tr>
<tr>
<td>2.</td>
<td>30</td>
<td>5.267</td>
<td>584223</td>
</tr>
<tr>
<td>3.</td>
<td>60</td>
<td>5.273</td>
<td>569807</td>
</tr>
<tr>
<td>4.</td>
<td>90</td>
<td>5.297</td>
<td>572215</td>
</tr>
<tr>
<td>5.</td>
<td>120</td>
<td>5.303</td>
<td>579153</td>
</tr>
</tbody>
</table>

### Table 4. Determined peak area for 4-chlorobenzoic acid for Solution 3 for the indicated time intervals.

<table>
<thead>
<tr>
<th>Number</th>
<th>Time (min)</th>
<th>Rt (min) 4-chlorobenzoic acid</th>
<th>Peak area of 4-chlorobenzoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0</td>
<td>5.101</td>
<td>567181</td>
</tr>
<tr>
<td>2.</td>
<td>30</td>
<td>5.208</td>
<td>578048</td>
</tr>
<tr>
<td>3.</td>
<td>60</td>
<td>5.267</td>
<td>584223</td>
</tr>
<tr>
<td>4.</td>
<td>90</td>
<td>5.273</td>
<td>569807</td>
</tr>
<tr>
<td>5.</td>
<td>120</td>
<td>5.303</td>
<td>579153</td>
</tr>
</tbody>
</table>
Table 4. Determined peak area for indomethacin and 4-chlorobenzoic acid for Solution 3 for the indicated time intervals.

<table>
<thead>
<tr>
<th>Number</th>
<th>Time</th>
<th>Rt (min)</th>
<th>Peak area of indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0</td>
<td>9.786</td>
<td>4286630</td>
</tr>
<tr>
<td>2.</td>
<td>30</td>
<td>9.841</td>
<td>198798</td>
</tr>
<tr>
<td>3.</td>
<td>60</td>
<td>9.869</td>
<td>33791</td>
</tr>
<tr>
<td>4.</td>
<td>90</td>
<td>9.878</td>
<td>19646</td>
</tr>
<tr>
<td>5.</td>
<td>120</td>
<td>9.883</td>
<td>14607</td>
</tr>
</tbody>
</table>

The results show that the incubation of indomethacin RS in buffer solution with pH 2.0 (close to stomach pH) and temperature of 37°C showed no changes in the peak area of the peak corresponding by retention time to that of the analyzed Indomethacin RS substance. Thus it may be concluded, that the analyzed substance could be considered stable under these conditions (Fig. 4).

A similar kinetic profile was obtained, when incubated in buffer with pH = 7.4 and 37°C temperature for the same time interval. The corresponding chromatographic profile and retention times for the analyzed substances are presented on Fig. 5.

The presented results that indomethacin may be considered stable in solutions with pH 7.4 and below, are confirmed by literary data [6].

The kinetic profile of the stability of indomethacin in buffer solution with pH = 9.0 was also followed, using the modified RP-HPLC method. A high decrease in the peak area of the peak corresponding by retention time to the analyzed Indomethacin substance is observed in the 30th min of incubation at 37°C (Fig. 6). In addition an increase in the peak area corresponding by retention time to the 4-chlorobenzoic acid is also observed (Fig. 6). Almost complete exhaustion of the initial indomethacin was obtained at the 60th min of the incubation.

The obtained chromatograms confirmed the expected results for rapid decomposition in alkaline solutions. From the performed experiments is visible, that indomethacin and its degradation product 4-chlorobenzoic acid are completely separated from each other, which indicates that the modified and validated proposed RP-HPLC method is selective and could be used for their simultaneous identification, quantitation and in purity testing.

The decrease in the measured peak areas observed in the chromatogram of Solution 3 shows, that hydrolysis occurs in the first 30 min of the incubation at the analyzed pH (pH = 9.0). On the other hand, no visible decrease in the peak areas was observed in Solution 1 and Solution 2 (1.2 for Solution 1 and pH = 7.4 for Solution 2), which indicates lack of hydrolysis at these conditions. This observation may be visualized with the following graph (Fig. 7).

From the followed formation of the degradation product 4-chlorobenzoic acid, monitored at constant pH of 9.0 and temperature of 37°C was found, that the process may be considered linear, indicating first-order degradation kinetics (Fig. 8).

![Fig. 4. Chromatographic profile of indomethacin RS of stability evaluation in acid media (pH = 2.0) at 0th min (a) and 120th min (b) of incubation under 37°C.](image-url)
Evaluation of the stability of indomethacin substance...

Fig. 5. Chromatographical profile of indomethacin RS of stability evaluation in acid media (pH = 7.4) at 0th min (a) and 120th min (b) of incubation under 37°C.

Fig. 6. Chromatograms obtained during the analysis of indomethacin RS in Solution 3 (buffer with pH 9.0) at 0th min (a) and at 30th min (b) of incubation under 37°C.

Fig. 7. Time dependence of the calculated concentrations for Solution 1, Solution 2 and Solution 3.

Fig. 8. First order plot of degradation of indomethacin in alkali media.
This result was further confirmed in the literature, where the apparent first-order rate constants have been calculated [7, 18]. It has been established, that the rate constant-hydroxide-ion concentration profile is linear with a negative slope and these experimental data confirmed the proposed reaction of degradation [7].

**Conclusion**

A fast, simple and fully automated analytical method for determination of indomethacin substance and its impurity 4-chlorobenzoic acid using RP-HPLC with UV detection was modified and validated. The chromatography was performed using isocratic elution with mobile phase composed of acetonitrile and 0.5% phosphoric acid (50:50, v/v) at flow rate 1.5 ml/min. 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 stability study of Indomethacin RS in conditions close to physiological.

A first order rate constant for the hydrolysis of indomethacin in alkali media was determined from the slope of linear plot of the logarithm of residual Indomethacin against time.

**References**


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