Isolation, Characterization of a Potential Degradation Product of Aspirin and an HPLC Method for Quantitative Estimation of Its Impurities

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In this work, a new degradation product of Aspirin was isolated, characterized and analyzed along with other impurities. New unknown degradation product referred as UP was observed exceeding the limit of ICH Q3B identification thresholds in the stability study of Aspirin and Dipyridamole capsule. The UP isolated from the thermal degradation sample was further studied by IR, Mass and 1H NMR spectrometry, revealing structural similarities with the parent molecule. Finally, UP was identified as a new compound generated from the interaction of Aspirin and Salicylic acid to form a dehydrated product. A specific HPLC method was developed and validated for the analysis of UP and other Aspirin impurities (A, B, C, E and other unknown degradation products). The proposed method was successfully employed for estimation of Aspirin impurities in a pharmaceutical preparation of Aspirin (Immediate Release) and Dipyridamole (Extended Release) Capsules.

Introduction

Aspirin (ASP) is chemically known as 2-acetyloxybenzoic acid (Acetysalicylic acid). ASP is extensively used in the treatment of mild-to-moderate pain, fever and inflammatory diseases (1). Dipyridamole (DPY) is an antiplatelet agent chemically described as 2,2’,2”-[(4,8-dipiperidinopyrimido[5,4-d] pyrimidine-2,6-diyl)dinitrilo]-tetraethanol [http://www.rxlist.com/aggrenox-drug.htm]. The combination of ASP and DPY is widely used to reduce thrombosis in patients with thrombotic diseases.

The chemical structure reveals that ASP is an ester moiety, which is very susceptible to hydrolysis under different hydrolytic conditions. ASP is known to undergo decomposition by hydrolysis into Salicylic acid, when exposed to moisture. It is reported that the decomposition reaction is promoted at high temperature, in alkaline solutions, even in the presence of magnesium salt (2). The known impurities of ASP are already reported, which includes salicylic acid, salsalate (salicylsalicylic acid), acetylsalicylsalicylic acid, 4-hydroxybenzoic acid, 4-hydroxyisophthalic acid and acetylsalicylsalicylic anhydride (3, 4). Salicylic acid is the only degradation product commonly reported in most of the literature. Other impurities are process-related impurities and generated during the synthesis of drug substance. The appearance of these process impurities depends on the process of synthesis and starting material used.

Stability testing forms an important part of the process of drug product development (5). The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time, under the influence of a variety of environmental factors such as temperature, humidity and light. The study also enables recommendation of storage conditions, retest periods and shelf life to be established. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved.

Many analytical approaches, such as liquid chromatography in combination with mass spectrometry (6, 7), ultraviolet detection (8–14), spectrophotometry method (15–17) and HPTLC method (18) are reported for the determination of ASP and its degradation products. However, so far no official report is available on the identification and characterization of ASP–salicylic acid degradation product. The characterization and quantitation of each impurity-related substance or degradation product present in the active substance or pharmaceutical formulation is regularly performed by HPLC-UV analytical techniques. It is particularly important to determine the UV response factor of the impurity, since it can be different from that of the active substance. In such cases, it could happen that the active substance is declared to be pharmaceutically confirmed according to the requirements, although the levels of its impurities may be outside the permitted values. Hence, it was felt necessary to identify and characterize the above unknown degradation product (UP) and develop a reliable, accurate method for the quantitative determination of UP along with other impurities in pharmaceutical formulation.

Experimental

Reagents and materials

ASP, DPY and impurities were obtained from Vergo Pharma Research Laboratories, Goa, India. Disodium hydrogen orthophosphate (AR grade), Acetonitrile (HPLC grade), Orthophosphoric acid were from Merck (Darmstadt, Germany). Water was purified by a Millipore (Bedford, MA, USA) Milli-Q water-purification system and passed through a 0.22 μm membrane filter before use.

Instrumentation

HPLC analysis was performed with an Agilent (Singapore) 1260 HPLC system equipped with a quaternary solvent manager, sample manager, column-heating compartment and photo diode array detector. This system was controlled by Agilent OpenLab software. An Inertsil ODS-3, 250 mm × 4.6 mm, 5 μm (GL Science, USA) was used for chromatographic separation. ASP
sample was degraded in Venticell Hot air oven (Germany). All samples were centrifuged by Thermo Scientific multifuged machine (GmbH, Germany). An API 2000™ LC–MS–MS System (AB SCIEX, Germany) triple quadrupole mass spectrophotometer was used for mass determination and 400 MHz NMR Spectrometer (Bruker, Germany) for proton NMR determination.

### Analytical HPLC method for impurity analysis and identification

An In-house LC gradient method was developed for the analysis of ASP impurities in ASP and DPY capsules using an Inertsl ODS-3 C18, (250 × 4.6)mm, 5 µm column with a mobile phase consisting of 0.01 M Na2HPO4, pH adjusted to 2.5 with orthophosphoric acid as mobile phase A. Mobile phase-B was acetonitrile. Composition of eluent was varied at a constant flow rate 1.0 mL/min and UV detection at 227 nm was used. The beginning ratio of mobile phase was A:B-67:33 (V/V) for 1 min and then the ratio was changed linearly 40:60 (V/V); within 20 min. The system came back to initial ratio at 40 min and continued at the same ratio up to 45 min. This LC method suitability was evaluated for separation of salicylic acid (Impurity-C), salsalate (Impurity-E), 4-hydroxybenzoic acid (Impurity-A), 4-hydroxyisophthalic acid (Impurity-B) and unknown impurities of ASP in ASP and DPY capsule. Stability sample of ASP and DPY capsule (40°C/75% RH, 3 months) was prepared at a concentration of 1 mg mL⁻¹ of ASP. The standard stock solution was prepared by dissolving an accurately weighed amount of ASP drug substance in mobile phase, resulting in a concentration of 5 µg mL⁻¹. An unknown degradation product (UP) identified at relative retention time (RRT) 2.9 was more than the permitted ICH limit (19) of 0.2% and increased significantly on storage.

### Purification and characterization of degradation product

UP crude sample was prepared by heating about 2 gm of drug substance at 120°C for 4 h to generate UP at the highest level for isolation. The above sample was dissolved in 5 mL of acetonitrile and diluted to 25 mL with a mixture of water and acetonitrile in the ratio of 1:1. The isolation of UP was carried out using a reversed-phase C18, 250 × 21.2 mm, 10 µm preparative column. Separation was carried out using water pH adjusted to 2.5 with orthophosphoric acid as mobile phase A and acetonitrile as mobile phase B. The separation was achieved by gradient elution at a constant flow rate 50.0 mL/min and the beginning ratio of mobile phase was A:B-60:40 (V/V); then the ratio changed linearly to 10:90 (V/V) within 11 min. The system came back to initial ratio at 17 min and continued at the same ratio for 3 min. UV detection was performed at 227 nm. The sample injection volume was 5 mL. The pure fractions were evaporated under reduced pressure, non-volatile buffer salts were removed by de-salting procedure and finally, fractions were lyophilized.

The purity and RRT of UP were confirmed in analytical HPLC method. The mass spectrum of isolated UP was obtained using the mass spectrometer (API 2000) equipped with electrospray ionization in negative mode. Samples were introduced into the mass spectrometer by direct infusion. ¹H NMR spectra were recorded in a Varian 400 MHz spectrometer. Typical 5–10 mg of sample was prepared in 0.70 mL DMSO for ¹H measurements. An infrared (IR) spectrum of UP was obtained by using Shmadzu attenuated total reflectance system. The spectra recorded were in the range of 400–4,000 cm⁻¹ by using 2 mg of sample.

### Validation of the method

The method was validated for specificity, sensitivity, linear range, accuracy, precision and robustness as per the International Conference on Harmonization (ICH) guidelines (20).

#### Specificity

A study was conducted to demonstrate the interference from placebo. Sample solutions were prepared by taking the placebo equivalent to the amount present in the sample solution and analyzed as per the test method. Chromatograms of placebo preparations are not showing any interference at the retention time of known impurities as well as analyte peaks. A study was conducted to demonstrate the known impurities interference by spiking the sample solution with all the known impurities at 0.3% spike level and analyzed as per the test method. It was found that all the known impurities separated from each other and also from the main analyte peaks.

The known impurities of ASP and DPY were injected individually to confirm the retention time.

A study was conducted to demonstrate the effective separation of degradants from ASP as well as from each other. The drug product was subjected to hydrolysis by refluxing the test solution in 0.1 N Sodium hydroxide solutions at 70°C for 4 h. Similarly, the acidic hydrolysis was performed by refluxing test solution in 0.1 N Hydrochloric acid solution at 70°C for 1 h. Oxidation studies were performed in 3% Hydrogen Peroxide solution at bench top for 1 h. On photo stability study, drug product was sufficient ly spread on petri plates (about 1 mm thick layer) and exposed to sunlight and UV light at ambient conditions for 7 days. Humidity study was performed separately by exposing drug product to humidity at 25°C, 90% RH for 5 days. Thermal degradation study was performed by heating drug product at 105°C for 1 h. Similarly, placebo samples were prepared as like as drug product by exposing formulation matrices without drug substance.

Stressed samples were injected into the HPLC system with photo diode array detector by following the test method conditions and peak purity evaluated.

#### Precision

The precision of the test method was evaluated by using six samples spiked with known impurities at 0.3% level and analyzed as per the test method.

#### Accuracy

To confirm the accuracy of the method, recovery studies were carried out by standard addition technique. Samples were prepared in triplicate by spiking all known impurities in test preparation at the level of limit of quantification (LOQ), 50, 100 and 150% of the limit concentration (2.0% for Salicylic acid and 0.3% for other impurities) and analyzed as per the test method.

#### Limit of detection and limit of quantification

Sensitivity of the method was established with respect to the limit of detection (LOD) and LOQ for ASP impurities. Series of concentration of drug solution and its impurities were injected and LOD and LOQ were established by signal-to-noise ratio method.
**Linearity of detector response**

A series of solutions of all the known impurities in the concentration ranging from LOQ to 150% of limit concentration were prepared and injected into the HPLC system.

**Real-time sample analysis**

The method suitability was verified by analyzing reference finished product and in-house formulated product. The content of 20 capsules were accurately weighed to determine average weight and transferred 50 mg equivalent of ASP into a 50 mL volumetric flask, about 30 mL of diluent added and kept for sonication for about 20 min with intermittent shaking, diluted to volume with diluent and mixed well. The solution was filtered through 0.45 µm PVDF filter and injected.

**Results**

**Characterization**

The accepted requirement for characterization of an organic compound includes establishment of purity, molecular weight, determination of functional group and elucidation of structural formula. The purity of UP was determined by HPLC method and found to be 98% pure.

To verify the structure of isolated UP, IR, MS and NMR experiments were performed. Mass of the compound was found to be 299 which is higher than the mass of ASP molecular weight (Figure 1). IR spectrum of compound is found quite similar in comparison to the spectra of ASP with slight difference in fingerprint region. Similarly, NMR spectra of the UP show 16 numbers of protons as compared to eight protons in ASP (Figure 2A and B).

**HPLC method development**

Selectivity, sensitivity, resolution and speed of chromatographic separation were optimized for the HPLC method. The retention times of Impurity A at about 4.39, B at 5.04, C at 11.10, Impurity E at 25.47, thermal degradation product (UP) at 23.71 and ASP at 7.80 min, respectively (Figure 3).

ASP and its impurities were well separated with good peak shape and resolution. No interfering peaks were observed in blank and placebo, indicating that signal suppression or enhancement by the product matrices was negligible.

**Method validation**

After satisfactory development of method, it was subjected to method validation as per the ICH guideline (20). The result of

![Figure 1. Mass spectra and fragmentation pattern.](https://academic.oup.com/chromsci/article-abstract/53/9/1491/307656)
system suitability parameter was found to be complying with the acceptance criteria: relative standard deviation of replicate injection is not more than 5.0% (Table I). All the impurities peaks are found separated from each other as well as from parent peak and peak purity passes in all stress conditions. The % RSD of replicate determination was found to be <5 during precision study. All the
results obtained in the recovery study were found within the range of 85–115%. LOD and LOQ concentrations were determined and LOQ was found to be less than the ICH reporting threshold. The calibration curves of all impurities were obtained by plotting the peak area of individual impurity versus concentration over the range of LOQ to 150% and correlation coefficient values were found to be ≥0.995. Refer Table I for method validation data.

The applicability of the method was verified by the determination of impurities in Aggrenox (Reference product) and in-house formulation of ASP and DPY capsules. Impurity profile data of both formulations has been given in Table II.

**Discussion**

**Structural analysis**

The Mass spectrum of UP is taken in negative mode and main molecular ion peak was 299 (m/z) as shown in Figure 1, which is 120 units more than ASP. The relative molecular mass of Salicylic acid is 138 and just 18 units more than the difference obtained between UP and ASP molecular ion peak (120 units). The mass spectrum data led us to suggest that structure could be a combination of ASP and Salicylic Acid with loss of one molecule of water. The spectra also showed a prominent peak at 257, which is due to the formation of stable fragment of UP as shown in Figure 1. The formation of a compound with above molecular weight was predicted based on degradation pathways of Aspirin (Figure 4) followed by structure confirmation with IR and NMR spectra. The IR spectra of the compound studied and identified band at 692 cm\(^{-1}\) corresponds to C=C aromatic ring bending, 740 cm\(^{-1}\) corresponds to C-H out of plane bend, stretching at 1,741 cm\(^{-1}\) corresponds to C=O in acetate carbonyl, 1,456 cm\(^{-1}\) bending corresponds to aliphatic C-H, stretching vibration at 1,681 cm\(^{-1}\) corresponds to C=O of carboxylic acid anhydride, stretching vibration at 1,606 cm\(^{-1}\) corresponds to aromatic C=C ring. We can conclude from the above data that IR spectra is supporting to the proposed structure as given in Figure 4. Similarly, we can identify from the \(^1\)H NMR spectra in Figure 3 that resonance at 8.17–8.19 and 7.97–7.99 ppm (doublet of doublet) corresponds to aromatic proton attached to C3 & C3’.

<table>
<thead>
<tr>
<th>Validation Parameters</th>
<th>Imp-A</th>
<th>Imp-B</th>
<th>Imp-C</th>
<th>Imp-E</th>
<th>Imp-I(UP)</th>
</tr>
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<tbody>
<tr>
<td>RRF value</td>
<td>0.39</td>
<td>0.64</td>
<td>0.89</td>
<td>1.20</td>
<td>0.70</td>
</tr>
<tr>
<td>LOQ in ppm</td>
<td>0.09</td>
<td>0.02</td>
<td>0.09</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Precision (% RSD)</td>
<td>0.4</td>
<td>0.2</td>
<td>0.5</td>
<td>0.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Accuracy (Min &amp; max % recovery)</td>
<td>91.0–97.8</td>
<td>94.3–102.7</td>
<td>92.3–107.6</td>
<td>102.5–103.6</td>
<td>95.3–105.2</td>
</tr>
<tr>
<td>Linearity from LOQ to 150% (n = 5)</td>
<td>180,738</td>
<td>1,209,267</td>
<td>413,281</td>
<td>576,711</td>
<td>19,006</td>
</tr>
<tr>
<td>Standard error</td>
<td>25,803</td>
<td>71,336</td>
<td>30,549</td>
<td>110,000</td>
<td>2,119</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>0.997</td>
<td>0.999</td>
<td>0.999</td>
<td>0.995</td>
<td>0.998</td>
</tr>
</tbody>
</table>

Table II: Impurity Profile of Marketed Products

<table>
<thead>
<tr>
<th>Product name</th>
<th>%Imp-A</th>
<th>%Imp-B</th>
<th>%Imp-C</th>
<th>%Imp-E</th>
<th>%Imp-I(UP)</th>
<th>%Single unknown</th>
<th>%Total</th>
</tr>
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<tbody>
<tr>
<td>In-house formulation</td>
<td>ND</td>
<td>ND</td>
<td>0.38</td>
<td>ND</td>
<td>0.24</td>
<td>0.01</td>
<td>0.63</td>
</tr>
<tr>
<td>Aggrenox</td>
<td>ND</td>
<td>ND</td>
<td>0.32</td>
<td>ND</td>
<td>0.04</td>
<td>0.01</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Table I: Method Validation Result

Figure 3. Typical chromatogram of aspirin and impurities.
singlet at 2.23 ppm assigned to three protons attached to \( \text{CH}_3 \) group at C7. Finally, spectral data of IR, NMR and Mass led us to assign the chemical name of unknown impurity as 2-(acetyloxy)benzoic 2-hydroxybenzoic anhydride and molecular formula \( \text{C}_{16}\text{H}_{12}\text{O}_6 \).

**Method validation**

The method was validated to demonstrate that it is suitable for its intended purpose by standard procedure to evaluate adequate validation characteristics. The result of system suitability and precision studies are well within acceptable limit. Similarly, the result of specificity study ascertained that the known impurities are separated from each other and also from ASP. There is no interference observed due to DPY and its impurity peaks. Spectral purity of all stressed samples was found pure. Sensitivity of the method was verified and the method is found to be linear, accurate and precise at the LOQ to the highest level. Method validation results ascertained that the method is suitable for the analysis of Aspirin impurities in Aspirin and Dipyridamole Tablets.

**Conclusion**

ASP and DPY drugs are official in the Pharmacopoeia (4, 21) but their combination product is not reported in any pharmacopoeia. Based on the literature survey, the unknown degradation product UP and official method for separation ASP impurities in “ASP and DPY capsules” has not yet been reported.

The present work is concerned with identification and characterization of new degradation product of ASP and estimation of all known and unknown impurities in pharmaceuticals preparation by a suitable HPLC method. The UP was isolated by a reversed-phase chromatography with 98% purity. The structure, molecular formula of the UP was assigned based on spectral data of IR, mass and NMR and identified as a new compound generated from the interaction of Aspirin and Salicylic acid to form a dehydrated product. A suitable reversed-phase HPLC method has been developed and validated as per the ICH guideline. The method was found to be suitable for the analysis of Aspirin impurities in ASP & DYP formulations.

Since, formation of Salicylic acid is very common in ASP drug substance and drug product at even normal storage condition, the probability of formation of discussed degradation product is very high. Hence, identification and characterization of degradation product will help accurate estimation of UP by using response factor.

**Acknowledgments**

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**References**

19. ICH; Impurities in New Drug Products (Q3BR2).
20. ICH; Validation of Analytical Procedure: Text Methodology (Q2(R1).