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Synthesis and standardization of an impurity of acetaminophen, development and validation of liquid chromatographic method

Cemil Caner Arıkan [a,b], Necla Kulabaş [c], İlkay Küçükgüzel [c,*]

[a] Department of Pharmaceutical Chemistry, Institute of Health Sciences, Marmara University, Kartal, 34865 İstanbul, Turkey
[b] Department of Analytical Development and Validation, Atabay Pharmaceuticals and Fine Chemicals Inc., Kadıköy, 34718 İstanbul, Turkey
[c] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Marmara University, Başıbüyük, 34854 İstanbul, Turkey

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ABSTRACT

One of the impurities of acetaminophen, *N,N'*-(oxydi-4,1-phenylene)diacetamide (ODAA), which is not specified in the organic impurities analysis method of acetaminophen by high performance liquid chromatography (HPLC) in American Pharmacopoeia Version 42 (USP 42), was synthesized, characterized and standardized. A new and optimized liquid chromatographic method for the determination of organic impurities of acetaminophen was developed using an ultra-high performance liquid chromatographic (UHPLC) system, which can separate this impurity. This new liquid chromatographic method has been optimized and validated for the simultaneous determination of acetaminophen related compound B, acetaminophen related compound C, acetaminophen related compound D, acetaminophen related compound J and ODAA, the organic impurities in acetaminophen drug substance. Acetaminophen was also subjected to stress-testing under acidic hydrolysis, alkaline hydrolysis, oxidative degradation, thermal degradation and photolytic degradation for 15 days. The impurity molecule, ODAA was synthesized using 4,4′-oxydianiline and acetic anhydride. The chemical structure of the synthesized ODAA molecule was confirmed by characterization studies. The potency of ODAA was found to be 99.64% as a result of the relevant analyses. The chromatographic separation was achieved on a C8 (150 mm × 2.1 mm; 2-μm particle size) reversed-phase column using a gradient elution, being solvent A: methanol-water-glacial acetic acid (50:950:1, v/v/v) and solvent B: methanol-water-glacial acetic acid (500:500:1, v/v/v) flowing at a rate of 0.2 mL/min. The limits of quantitation (S/N 10:1) were 1.248 μg/mL for acetaminophen, 0.373 μg/mL for acetaminophen related compound B, 1.217 μg/mL for acetaminophen related compound C, 0.369 μg/mL for acetaminophen related compound D, 0.125 μg/mL for acetaminophen related compound J and 0.373 μg/mL for ODAA. The individual mean recoveries of each impurity molecule spiked into acetaminophen samples at different concentration levels ranged from 93% to 104%. The method developed for UHPLC instrument was successfully applied to the analyses of different lots of acetaminophen. Thus, the proposed method can be used for determination of this impurity in the presence of other specified impurities of acetaminophen.

1. Introduction

Organic impurities that may appear in acetaminophen (Table 1) are process and/or drug-related impurities and they may arise during the manufacturing process and/or storage of the drug substance. Organic impurity profiles are influenced by starting materials, choice of synthetic route, by-products, intermediates, degradation products, reaction conditions, reagents, ligands and catalysts, working conditions and final purification steps [1,2]. The presence of impurities in the pharmaceuticals even in small amounts may influence the efficacy and safety of the pharmaceutical products. Therefore, the identification as well as the quantification of impurities in the pharmaceuticals are the subject of considerable attention by both the manufacturer and regulatory authorities [3,4].

When acetaminophen is analyzed by HPLC according to the organic impurities analysis method of acetaminophen in American Pharmacopoeia Version 42 (USP 42) [5], an unknown impurity has been observed on the chromatogram which is not specified by the USP 42. The present compendial monographs for acetaminophen (American Pharmacopeia, European Pharmacopeia, or British Pharmacopeia) do not include HPLC methods for the potential impurities most likely to arise during the syntheses. These impurities may contribute to the impurity profile of the

* Corresponding author.
E-mail addresses: ikucukguzel@marmara.edu.tr, ilkay@kucukguzel.com (İ. Küçükgüzel).

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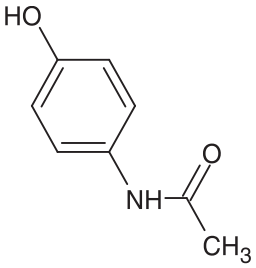
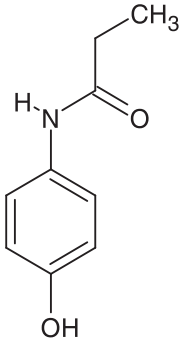
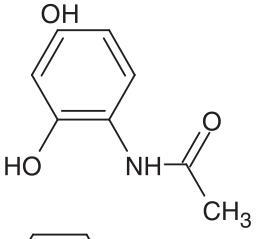
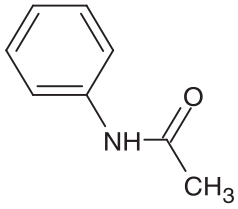
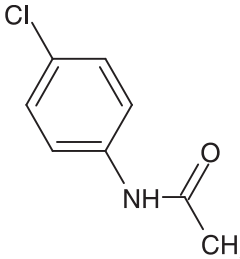
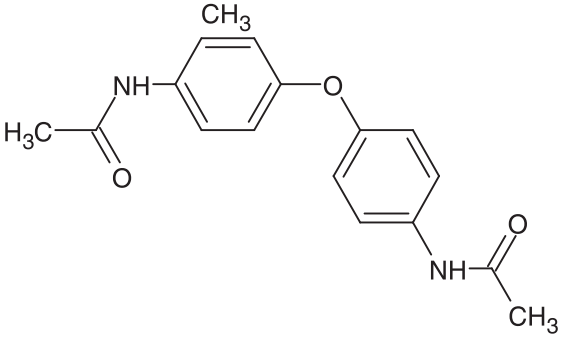
drug substance. Therefore, an analytical method development to go along with process design is needed to identify and quantify these impurities [3]. In literature, many analytical methods with different analytical methodologies and techniques have been proposed for the determination of various organic impurities of acetaminophen [2,3, 6–14]. However, no studies regarding simultaneous HPLC or UHPLC determination of organic impurities of acetaminophen and this unknown impurity have been published so far.

Table 1
Chemical structure of acetaminophen, its specified impurities and ODAA.

Name	Structure	Chemical name
Acetaminophen		*N*-(4-Hydroxyphenyl)acetamide
Acetaminophen related compound B		*N*-(4-Hydroxyphenyl)propanamide
Acetaminophen related compound C		*N*-(2-Hydroxyphenyl)acetamide
Acetaminophen related compound D		*N*-Phenylacetamide
Acetaminophen related compound J		*N*-(4-Chlorophenyl)acetamide
ODAA		*N*,*N*′-(Oxydi-4,1-phenylene)diacetamide

In the present work, this newly identified impurity molecule was synthesized, characterized, standardized and the current HPLC method in USP 42 for the analysis of organic impurities of acetaminophen was transferred to UHPLC by developing a new related method including this impurity as well. The new method developed for UHPLC instrument was validated according to the international conference on harmonization (ICH) guideline [15] and the stress-testing studies of acetaminophen were performed with forced degradation studies. The optimized method was applied on different lots of acetaminophen samples for routine application.

2. Experimental

2.1. Chemicals, reagents and samples

4,4′-Oxydianiline and acetic anhydride were of analytical grade provided by Atabay Pharmaceuticals and Fine Chemicals (Istanbul, Turkey). Acetone, ether, ethanol (absolute) and triethylamine were of analytical grade from Merck company (Darmstadt, Germany) provided by Marmara University (Istanbul, Turkey). Different lots of acetaminophen raw material and acetaminophen working standard were provided by Atabay Pharmaceuticals and Fine Chemicals (Istanbul, Turkey). The reference standard for acetaminophen related compound B was obtained from LGC Standards (Teddington, UK). Reference standards for acetaminophen related compound C and acetaminophen related compound D were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The reference standard for acetaminophen related compound J was obtained from Merck company (Darmstadt, Germany). HPLC grade methanol, UHPLC-MS grade methanol, glacial acetic acid (100%), sulfuric acid (98%), dimethyl sulfoxide (DMSO), hydrochloric acid 37% (HCI), sodium hydroxide (NaOH) and hydrogen peroxide 30% (H_2O_2) were purchased from Merck company (Darmstadt, Germany). HPLC grade distilled water was provided by Atabay Pharmaceuticals and Fine Chemicals (Istanbul, Turkey).

2.2. Liquid chromatography–mass spectrometry

The mass spectra were recorded on Shimadzu LC-MS/MS-8030 system (Shimadzu Corporation, Kyoto, Japan). The instrument was operated using positive electrospray ionization (ESI) mode with nebulizing gas flow of 2.8 L/min, drying gas flow of 15 L/min, DL temperature of 250 °C, heat block temperature of 400 °C, CID gas at 230 kPa, collision energy of − 5.0 V, scan speed of 15000 u/sec and event time of 0.030 s. Chromatographic separation was achieved on a Shim-Pack FC ODS (150 mm × 2 mm; 3.5-μm particle size) reversed-phase column (Shimadzu Corporation, Kyoto, Japan) maintained at 40 °C, using a gradient elution (Table S1), being solvent A: methanol-water-glacial acetic acid (50:950:1, v/v/v) and solvent B: methanol-water-glacial acetic acid (500:500:1, v/v/v), and methanol as diluent. The total pump flow rate was 0.25 mL/min and the injection volume was 1 μL. The ODAA sample was prepared at a concentration of 12.5 μg/mL in methanol.

Mass analysis was carried out by applying scan mode, followed by product ion scan mode. The selected ion in the product ion scan mode was split into smaller fragments by collision induced dissociation (CID) and the resulting fragments were analyzed.

2.3. HPLC

Chromatographic separations of acetaminophen and its related substances were performed on the Shimadzu LC-2040 C 3D Plus HPLC system equipped with a photodiode array detector and LabSolutions data handling system (Shimadzu Corporation, Kyoto, Japan) according to the monograph of acetaminophen in USP 42 [5]. HPLC analyses for characterization studies of ODAA and HPLC area normalization analysis for determining the potency of ODAA were performed on a Kromasil C8 (250 mm × 4.6 mm; 5-μm) reversed-phase column maintained at 40 °C. The separation was employed using gradient elution as indicated in Table S2. Details of the method and preparation of mobile phase, system suitability, standard and sample solutions are described in Section S.1. (Suppl. Mat.).

2.4. Gas chromatography

Gas chromatography (GC) analysis was performed on the Shimadzu GC-2010 Plus GC equipped with flame ionization detector (FID) and Shimadzu HS-20 headspace sampler instruments (Shimadzu Corporation, Kyoto, Japan) for determining the content of residual solvents in ODAA sample. The GC analysis was carried out on RTX-624 (30 m, 0.32 mmID 1.8 μm) column at 50 °C. FID temperature was 270 °C. The separation was employed using the gradient column temperature program given in Table 2. Details of the GC method and preparation of standard and sample solutions are described in Section S.2. (Suppl. Mat.).

2.5. Liquid chromatographic method using UHPLC instrument

Chromatographic separations for acetaminophen, acetaminophen related compound B, acetaminophen related compound C, acetaminophen related compound D, acetaminophen related compound J and ODAA were performed on the Shimadzu Nexera X2 UHPLC system equipped with a photodiode array detector and LabSolutions data handling system (Shimadzu Corporation, Kyoto, Japan). In this method, the analyses were carried out on InertSustain C8 (150 mm × 2.1 mm; 2-μm) reversed-phase column at 40 °C column temperature. The injection volume and detection wavelength were fixed at 1 μL and 254 nm, respectively. Data was acquired during 30 min by using gradient elution system flowing at a rate of 0.2 mL/min (Table 3). Mixture of methanol-water-glacial acetic acid (50:950:1, v/v/v) was used as solvent A; mixture of methanol-water-glacial acetic acid (500:500:1, v/v/v) was used as solvent B; and methanol as diluent. Details of the preparation of standard, sample and system suitability solutions are described in Section S.3. (Suppl. Mat.).

2.6. Validation of optimized liquid chromatographic method

The developed LC method using the UHPLC instrument was validated according to ICH Q2(R2) Validation of Analytical Procedures: Text and Methodology [15] with respect to system suitability, specificity, precision (system precision, method precision and intermediate precision), accuracy (recovery), linearity and range, robustness and solution stability. Validation studies were performed on the Shimadzu Nexera X2 UHPLC system equipped with a photodiode array detector and LabSolutions data handling system (Shimadzu Corporation, Kyoto, Japan).

2.7. Stress-testing

Stress-testing was performed on the Shimadzu Nexera X2 UHPLC system equipped with a photodiode array detector and LabSolutions data handling system (Shimadzu Corporation, Kyoto, Japan). The test was done to determine whether ODAA is a degradation or a synthesis impurity of acetaminophen. Acetaminophen was subjected to stress-testing under the following conditions: acidic hydrolysis, alkaline hydrolysis, oxidation degradation, thermal degradation and photolytic degradation under UV-light. Details of the stress-testing and preparation

Table 2
Gradient column temperature program in GC.

Rate	Temperature	Hold Time
	(°C)	(min)
–	50.0	10.0
60.00	240.0	3.0

Table 3
Gradient elution program of mobile phase in UHPLC.

Time	Flow	Solvent A	Solvent B
(min)	(mL/min)	(%)	(%)
0	0.2	65	35
1	0.2	65	35
6	0.2	22	78
22	0.2	22	78
23	0.2	65	35
30	0.2	65	35

of test solutions are described in Section S.4. (Suppl. Mat.).

2.8. Synthesis and standardization of ODAA

ODAA was synthesized by acetylation of 4,4′-oxydianiline using acetic anhydride [16]. Details of the synthesis reaction of ODAA were given in Section S.5. (Suppl. Mat.). Following the characterization studies, standardization of the synthesized ODAA was conducted by HPLC, GC, residue on ignition (sulfated ash) and water determination studies. Organic impurities were determined by HPLC analysis; inorganic impurities were determined by residue on ignition (sulfated ash) test; residual solvents were determined by GC analysis; water amount was determined by Karl Fischer test method, and thus, the total impurity amount in ODAA was determined.

3. Results and discussion

3.1. Identification of ODAA in acetaminophen batches and its subsequent synthesis and characterization

It all started when we saw a new and unknown impurity peak in the acetaminophen production batches that was not compatible with the authentic standards. Investigation of the LC-MS/MS data for this peak

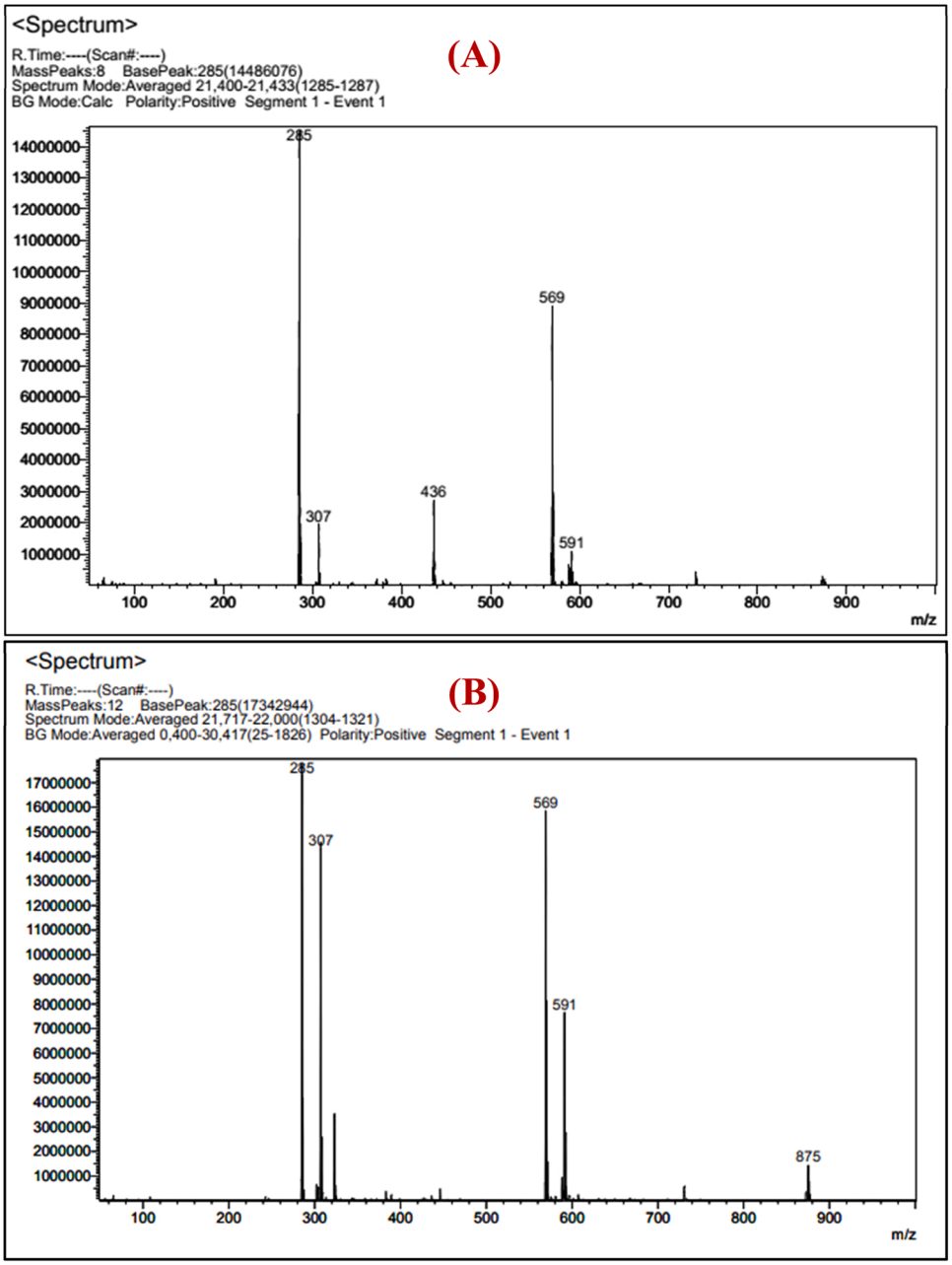


Fig. 1. Mass spectra of ODAA obtained by scan mode in LC-MS/MS: (A) impurity detected in acetaminophen batches, (B) synthesized impurity molecule.

revealed that, the molecular weight of this molecule was 284 as evident from a molecular ion peak (MH^+) at *m/z* 285 (Fig. 1). Structure of this compound was estimated as *N*,*N*′-[oxydi(4,1-phenylene)]diacetamide (ODAA) based on its molecular weight and its fragmentation data which was obtained in the product ion scan mode (Fig. 2). Then, we decided to synthesize this compound in order to use as a reference standard in our study. Preparation of ODAA was performed by the acetylation of 4,4′-oxydianiline using acetic anhydride as described [16].

Thin-layer chromatography (TLC) results showed that the synthesis reaction of ODAA completed successfully. ODAA was obtained as white crystals with overall 95% yield. Characterization of the synthesized ODAA was performed by melting point (Section S.6., Suppl. Mat.), FT-IR (Section S.7., Suppl. Mat.), HPLC, LC-MS/MS, ^{1}H NMR, ^{13}C NMR and elemental analysis studies. The melting point of the synthesized ODAA was found to be 230 °C showing conformity with its theoretical melting temperature stated in literature [17]. Elemental analysis results showed that the synthesized ODAA molecule was consisted of 66.89% C, 5.582% H and 9.849% N supporting the molecular formula of ODAA. The FT-IR spectrum of ODAA showed characteristic vibrational bands for N-H stretching, aromatic C-H stretching and aliphatic C-H stretching at 3279 cm^{-1}, 3194–3140 cm^{-1} and 3067 cm^{-1}, respectively. Vibrational bands at 1655 and 1612 cm^{-1} were assigned to C═O and C═C stretching, respectively. The N-H bending band appeared at 1551 cm^{-1}. Asymmetrical bending band in C-H bond appeared at 1500 cm^{-1}. Absorption bands at 1366–1315 cm^{-1} were assigned to symmetrical bending in C-H (Fig. S1). The peak observed at 45.993 min in the HPLC chromatogram of acetaminophen sample (Fig. S2) was demonstrated to be ODAA which showed a similar retention time as 45.976 min in its individual HPLC chromatogram (Fig. S3).

LC-MS/MS analysis of the synthesized ODAA was carried out in two different modes: scan and product ion scan. The *m/z* value for both the unknown impurity (Fig. 1A) and the synthesized ODAA (Fig. 1B) was found at 285 Da by scan mode. In the spectrum, it was also observed that dimer formation of ODAA gave a peak at *m/z* 569 (284 + 284 + 1 H^+). Further mass analysis of the precursor ion of 285 g/mol (*m/z*) of ODAA by product ion scan mode gave fragments that supported the obtained *m/z* value of ODAA. The mass spectra for the unknown impurity and the synthesized ODAA gave the completely similar fragmentation pattern (Fig. 1 and Fig. 2).

While the spectra of ^{1}H NMR (Fig. S4), ^{13}C NMR (Fig. S5) and HMBC NMR (Fig. S6) are given in Supplementary Material file, the findings are given in the Table 4. In the HMBC spectrum of ODAA, the -CH_3 (δC =23 ppm) group were detected as two symmetrical contours in the field corresponding to their own protons (δH=2.03 ppm). At the same time, it is possible to see the correlations between -C═O (δC =170 ppm) and -CH_3 (δH=2.03 ppm). When the zoomed HMBC spectrum of ODAA in the range of 6.5–8.0 ppm is examined, the correlations between the carbons of carbonyl group (δC= 152.79, for C1 and C11) and protons in both ortho (C2, C6, C10, C12) and meta (C3, C5, C7, C9) positions of the phenyl ring were detected at 6.9 and 7.6 ppm, respectively. Additionally, the detected correlations between -NH (δH =9.93 ppm) and -C═O group (δC=168.47 ppm), C4 and C8 carbons (δC=135.32) and C3, C5, C7 and C9 carbons (δC= 121.08 ppm) confirmed the structure of ODAA.

3.2. Standardization of ODAA

The HPLC chromatogram of the synthesized ODAA obtained by HPLC area normalization (area %) method is shown in Fig. S7. In the chromatogram, the retention time of the synthesized ODAA was found as 46.018 min and the area % of ODAA was found as 99.990%. According to this result, it was determined that 0.01% of organic impurity was present in the synthesized ODAA. As a result of the residue on

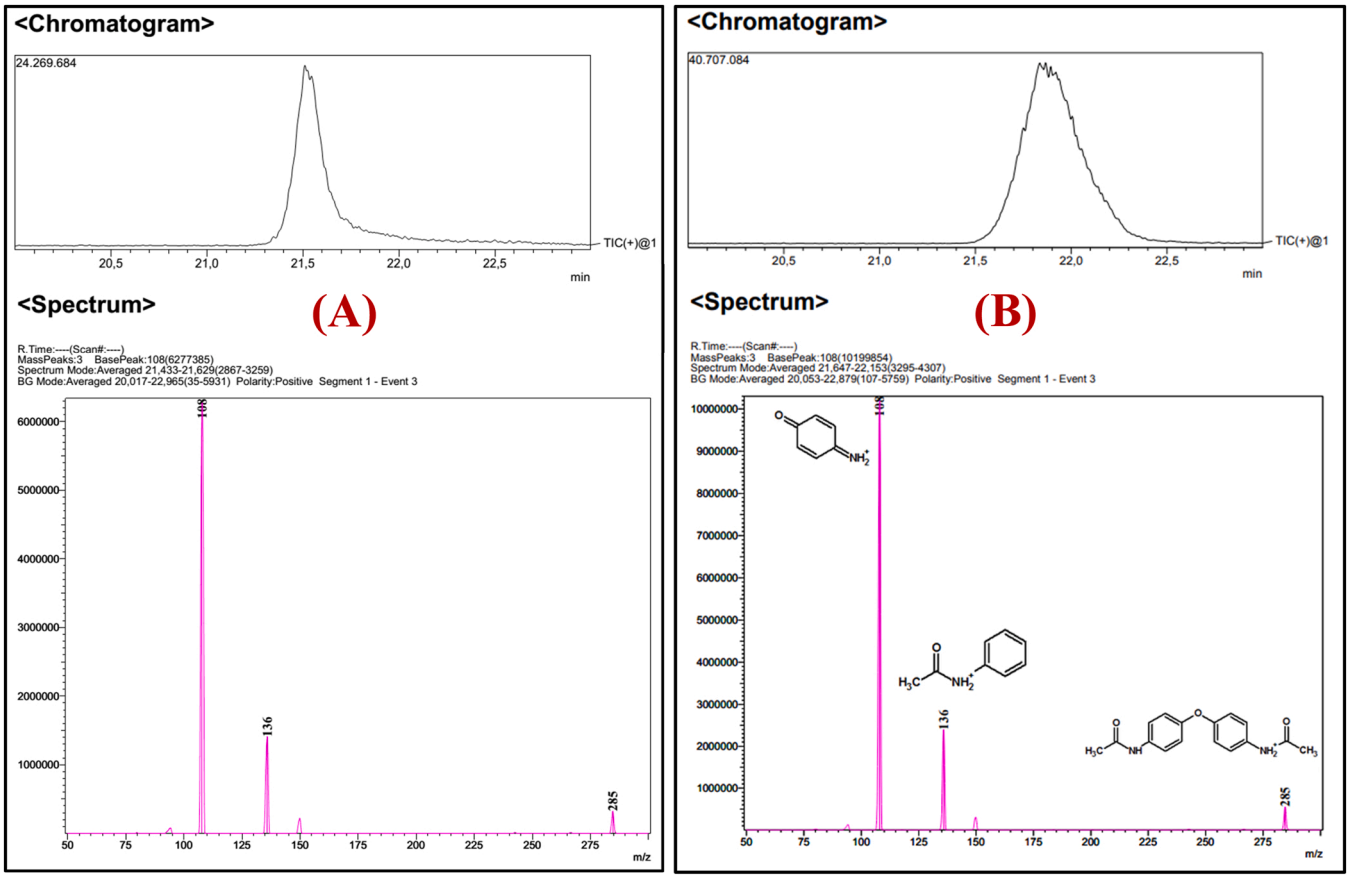
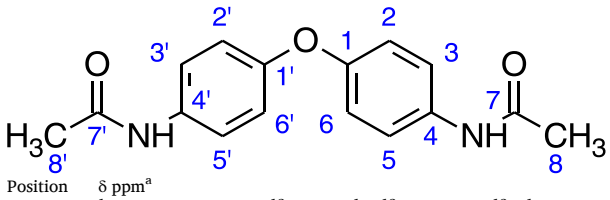


Fig. 2. Mass spectra of ODAA obtained by product ion scan mode in LC-MS/MS: (A) impurity detected in acetaminophen batches, (B) synthesized impurity molecule.

Table 4
^{1}H, ^{13}C and HMBC NMR assignments for ODAA.

Structure



Position	δ ppm[a] ^{1}H	^{13}C	^{1}H–^{13}C relations	^{13}C–^{1}H relations
1, 1′	–	152.79	–	2, 3, 5, 6, 2′, 3′, 5′, 6′
2, 2′	6.93 (4 H, d, Ar-H, *J*=9.0 Hz)	119.07	1, 2 * , 3, 4, 5, 6 * , 1′, 2′* , 3′, 4′, 5′, 6′*	2 * , 3, 5, 6 * , 2′* , 3′, 5′, 6′*
3, 3′	7.57 (4 H, d, Ar-H, *J*=9.0 Hz)	121.08	1, 2, 3 * , 4, 5 * , 6, 1′, 2′, 3′* , 4′, 5′* , 6′	2, 3 * , 5 * , 6, 2′, 3′* , 5′* , 6′, NH
4, 4′	–	135.32	–	2, 3, 5, 6, 2′, 3′, 5′, 6′, NH
5, 5′	7.57 (4 H, d, Ar-H, *J*=9.0 Hz)	121.08	1, 2, 3 * , 4, 5 * , 6, 1′, 2′, 3′* , 4′, 5′* , 6′	2, 3 * , 5 * , 6, 2′, 3′* , 5′* , 6′, NH
6, 6′	6.93 (4 H, d, Ar-H, *J*=9.0 Hz)	119.07	1, 2 * , 3, 4, 5, 6 * , 1′, 2′* , 3′, 4′, 5′, 6′*	2 * , 3, 5, 6 * , 2′* , 3′, 5′, 6′*
7, 7′	–	168.47	–	8, 8′, NH
8, 8′	2.032 (6 H, s, CH_3,)	24.32	7, 7′, 8 * , 8′*	8 * , 8′*
NH	9.93 (2 H, s, NH)	–	3, 4, 5, 7, 3′, 4′, 5′, 7′	–

[a] Spectra recorded in DMSO-*d6*. Assignments: s: singlet; d: doublet.

ignition (sulfated ash) test (Section S.8., Suppl. Mat.), it was determined that 0.009946% of inorganic impurity was present in the synthesized ODAA. According to the GC results, a total amount of 0.037027% of residual solvent was found in the synthesized ODAA containing 0.035527% of ethanol, 0.001328% of acetone and 0.000172% of triethylamine. Water determination by Karl Fischer test method (Section S.8., Suppl. Mat.) gave an average amount of water as 0.305% in the ODAA sample. All results obtained for determining the potency of the synthesized ODAA are summarized in Table S3.

The potency of the synthesized ODAA was calculated using the obtained results and the formula given in the literature [18,19] and found to be 99.64%. Thus, ODAA molecule was used as a reference standard.

3.3. Validation of the newly developed LC method

3.3.1. System suitability

System suitability was checked from the six replicate injections of the standard solution. The test was conducted throughout the validation study and the suitability requirements were expected to be met before each validation parameter. The acceptance criteria for the system suitability were as described in the supplemantary material (Section S.3., Suppl. Mat.). The results of the system suitability test in comparison with the required limits are shown in Table S4. According to the results presented, the proposed method using UHPLC instrument fulfills the requirements within the accepted limits.

3.3.2. Specificity

Specificity test was conducted to demonstrate whether the proposed analytical procedure is specific for the analytes to be examined. All solutions used in the developed LC method (diluent, solvent A, solvent B, standard solutions) were prepared individually and/or mixed, and analyzed in the UHPLC system. A mixture standard solution containing all analytes was prepared by mixing acetaminophen, acetaminophen related compound B, acetaminophen related compound C, acetaminophen related compound D, acetaminophen related compound J and ODAA. The individual UHPLC chromatograms of analytes and solvents were checked for retention times and relative retention times of peaks with respect to the main peak of acetaminophen. The UHPLC chromatogram of the mixture standard solution was checked if there was any chromatographic interference between the analytes (Fig. 3).

As a result of the specificity test, no overlapping peak was observed on the chromatograms of diluent, solvent A and solvent B at the retention times of the peaks of acetaminophen and its impurities. In the UHPLC chromatogram of the mixture standard solution containing all analytes, it was observed that none of the compounds interfered with each other, demonstrating that the proposed method could chromatographically separate acetaminophen and its impurities from each other successfully. The individual UHPLC chromatograms obtained for acetaminophen and its impurities were also assessed for the peak purities, and as a result all molecules were found homogeneous and spectrally pure (single point threshold < peak purity index). Thus, the developed method was found specific for determination of organic impurities in acetaminophen. The results of the specificity test are shown in Table 5.

3.3.3. Precision

The precision of the proposed method were assessed as system precision (repeatability), method precision and intermediate precision. The system precision was performed by making six replicate injections of the standard solution. Results obtained from repeatability test were assessed for RSD% values of peak areas and RSD% values of retention times. As can be seen in Table S5, all RSD% values for peak areas and retention times were found below the accepted limit of 5.0% for each analyte.

The method precision was investigated by injecting six individual sample solutions of acetaminophen each spiked with impurities of acetaminophen at their 100% limit concentration levels. The method precision at the LOQ levels was also investigated by injecting six individual sample solutions of acetaminophen each spiked with impurities of acetaminophen at their LOQ concentration levels. Confidence intervals (CI) were calculated at 95% confidence level. Results obtained from method precision test were assessed for RSD% values between assay % results for each impurity. As can be seen in Table S5, all RSD% values for assay % results assessed separately for LOQ and 100% levels were found below the accepted limit of 5.0% for each impurity.

The intermediate precision of the method was investigated by conducting method precision analysis on a different day. The results obtained from the intermediate precision analysis performed both at LOQ and 100% levels are shown in Table S6 and Table S7, respectively. Confidence intervals (CI) were calculated at 95% confidence level. The RSD% values between the twelve assay % results obtained from day-1 and day-2 were found below the accepted limit of 5.0% for each impurity, both at LOQ and 100% levels. As a result of the precision study, the developed method was demonstrated to be precise.

3.3.4. Accuracy (Recovery)

Accuracy of the proposed method was conducted on acetaminophen samples spiked with known amounts of impurities of acetaminophen and the samples were analyzed in the UHPLC system. The study was assessed using 12 determinations over 4 concentration levels (LOQ, 50%, 100% and 150%) of each impurity of acetaminophen with 3 replicates for each level. Confidence intervals (CI) were calculated at 95% confidence level. Results obtained from recovery studies are given in Table 6. The accuracy experiments showed mean recoveries of 101.03%, 97.30%, 96.87%, 99.29% and 97.52% with RSD% values of 1.10%, 2.60%, 2.41%, 2.73% and 1.99% for acetaminophen related compound B, acetaminophen related compound C, acetaminophen related compound D, acetaminophen related compound J and ODAA, respectively. All recovery results were found between 90.0% and 110.0% with RSD%

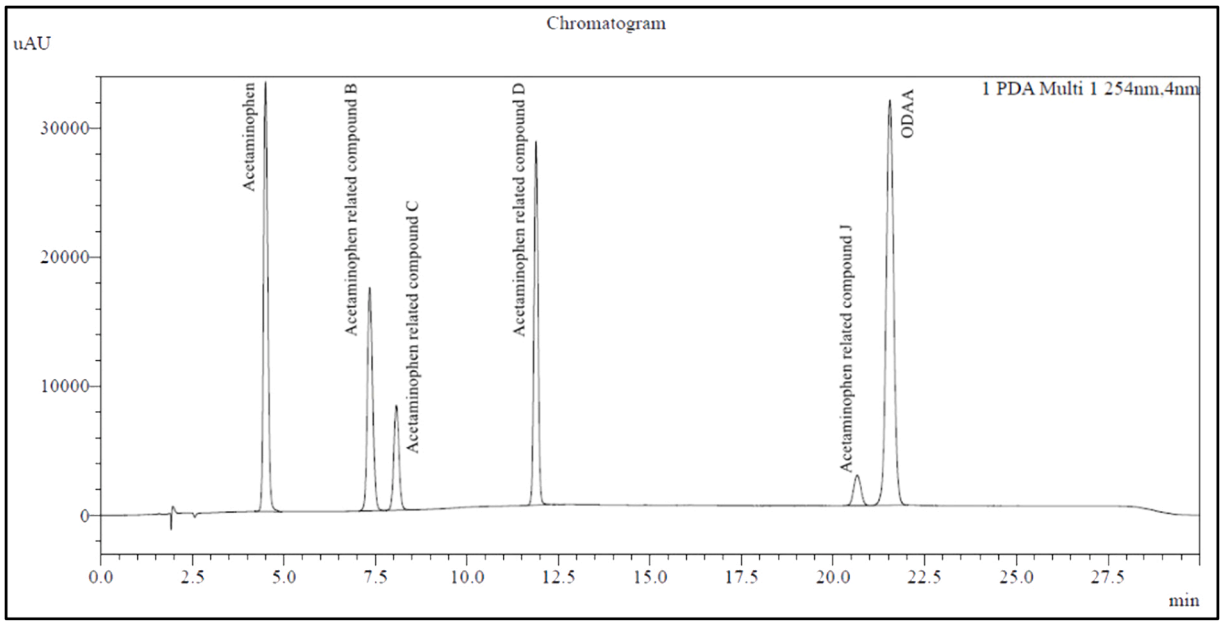


Fig. 3. UHPLC chromatogram of the mixture standard solution.

Table 5
Specificity results of the developed method and the impurity limits of acetaminophen.

Substance	Limit (max. %)	Retention Time (RT) (min)	Relative Retention Time (RRT)	Peak Purity Index	Single Point Threshold
Acetaminophen	–	4.492	1.000	1.0000	0.9415
Acetaminophen related compound B	0.050	7.339	1.634	1.0000	0.7892
Acetaminophen related compound C	0.050	8.061	1.795	1.0000	0.8700
Acetaminophen related compound D	0.050	11.881	2.645	1.0000	0.9560
Acetaminophen related compound J	0.001	20.649	4.597	1.0000	0.8352
ODAA	0.050	21.533	4.794	1.0000	0.9187
Individual unspecified impurity	0.050	–	–	–	–
Total impurities*	0.100	–	–	–	–

* : not the total of all impurities.

values of $\leq$ 5.0%, and the bias values were found within the limits of $\pm$ 15%, demonstrating that the developed method is accurate.

3.3.5. *Linearity, range, limit of detection (LOD) and limit of quantitation (LOQ)*

Linearity data were obtained using standard solutions containing acetaminophen, acetaminophen related compound B, acetaminophen related compound C, acetaminophen related compound D and ODAA, prepared at eight different concentration levels ranging from 1% to 200% of their limit concentrations. Linearity for acetaminophen related compound J was assessed individually at six different concentration levels ranging from 20% to 200% of its limit concentration since its limit concentration is different from that of other impurities. Triplicate injections were made for each concentration level. Thus, the range for each molecule was determined starting from their individual LOQ values up to 200% of their limit concentrations.

Calibration curves for acetaminophen, acetaminophen related compound B, acetaminophen related compound C, acetaminophen related compound D, acetaminophen related compound J and ODAA were constructed by plotting the peak area response against the concentration. The peak area response versus concentration data was analyzed with least squares linear regression. All coefficient of determination values (r^2) in regression equations for all substances were found to be greater than 0.99 indicating the good linearity of the method. The regression coefficients of all equations were statistically evaluated by t-test. t_{Stat} values were compared with $t_{Critical}$ values and all t_{Stat} values were found to be greater than $t_{Critical}$ values. The results showed that all regression coefficients were statistically significant at 95% confidence ($p > 0.05$) of the data sets (Table S8).

The limits of quantitation (S/N 10:1) were found 1.248 μg/mL for acetaminophen, 0.373 μg/mL for acetaminophen related compound B, 1.217 μg/mL for acetaminophen related compound C, 0.369 μg/mL for acetaminophen related compound D, 0.125 μg/mL for acetaminophen related compound J and 0.373 μg/mL for ODAA. All obtained data from linearity, range, LOD and LOQ studies are summarized in Table 7.

3.3.6. *Robustness*

Robustness of the developed method was tested by analyzing sample and standard solutions under small and deliberately varied chromatographic conditions to measure the reliability of the method. Sample solutions of acetaminophen were spiked with impurities of acetaminophen at their limit concentrations and standard solutions for each analysis were prepared as described in Section S.3. (Suppl. Mat.). Chromatographic conditions were altered by changing the wavelength by $\pm$ 2 nm (254 $\pm$ 2 nm), and the column temperature by $\pm$ 5 °C (40 $\pm$ 5 °C). Only one change was made at a time. System suitability parameters for each four varied chromatographic condition were checked from the standard solution and all parameters have been observed within the acceptance criteria. Results of the sample solutions analyzed at different chromatographic conditions showed no significant difference in comparison to the results obtained under normal method conditions. RSD% values between the results obtained under normal and altered method conditions are given in Table S9. All RSD% values were found below the accepted limit of 5.0%, demonstrating that the developed method could remain unaffected by small and deliberate variations in method parameters.

Table 6
Accuracy results of the developed method.

Substance	Concentration (Mean, μg/mL) (n = 3)		Recovery (Mean, %)	RSD (Mean, %)	Bias (Mean, %)
	Theoretical	Found ± CI (α = 0.05)	(n = 3)	(n = 3)	(n = 3)
LOQ Level					
Acetaminophen related compound B	0.3722	0.3760 ± 0.0066	101.02	0.704	1.021
Acetaminophen related compound C	1.1980	1.2123 ± 0.0137	101.20	0.454	1.196
Acetaminophen related compound D	0.3684	0.3663 ± 0.0174	99.44	1.917	-0.561
Acetaminophen related compound J	0.1250	0.1247 ± 0.0014	99.73	0.463	-0.267
ODAA	0.3729	0.3673 ± 0.0057	98.51	0.629	-1.493
50% Level of the limit concentration					
Acetaminophen related compound B	6.2213	6.2559 ± 0.0442	100.56	0.284	0.556
Acetaminophen related compound C	6.0868	5.7633 ± 0.0333	94.69	0.232	-5.313
Acetaminophen related compound D	6.1529	5.7705 ± 0.0499	93.79	0.348	-6.214
Acetaminophen related compound J	0.1241	0.1206 ± 0.0061	97.15	2.036	-2.847
ODAA	6.2126	5.8907 ± 0.0343	94.82	0.234	-5.181
100% Level of the limit concentration					
Acetaminophen related compound B	12.4426	12.4698 ± 0.2970	100.22	0.959	0.219
Acetaminophen related compound C	12.1735	11.7620 ± 0.1355	96.62	0.464	-3.381
Acetaminophen related compound D	12.3057	11.9742 ± 0.2167	97.31	0.728	-2.694
Acetaminophen related compound J	0.2482	0.2557 ± 0.0027	103.04	0.432	3.035
ODAA	12.4251	12.3578 ± 0.2644	99.46	0.861	-0.542
150% Level of the limit concentration					
Acetaminophen related compound B	18.6639	19.1014 ± 0.5427	102.34	1.144	2.344
Acetaminophen related compound C	18.2603	17.6591 ± 0.3213	96.71	0.732	-3.292
Acetaminophen related compound D	18.4586	17.8973 ± 0.4998	96.96	1.124	-3.041
Acetaminophen related compound J	0.3723	0.3620 ± 0.0117	97.22	1.300	-2.776
ODAA	18.6377	18.1341 ± 0.5557	97.30	1.234	-2.702
Substance			Recovery (Mean, %) (n = 12)	RSD (Mean, %) (n = 12)	Bias (Mean, %) (n = 12)
Acetaminophen related compound B			101.03	1.100	1.035
Acetaminophen related compound C			97.30	2.600	-2.697
Acetaminophen related compound D			96.87	2.407	-3.128
Acetaminophen related compound J			99.29	2.733	-0.714
ODAA			97.52	1.990	-2.479

Table 7
Results of regression analysis of the linearity data of acetaminophen and its impurities.

Substance	Range	Slope	Intercept	r^2	LOD	LOQ
	(μg/mL)				(μg/mL)	(μg/mL)
Acetaminophen	1.248–24.960	18082420	4345.691	0.9998	0.374	1.248
Acetaminophen related compound B	0.373–24.885	13226230	415.7887	0.9994	0.124	0.373
Acetaminophen related compound C	1.217–24.347	6015866	324.0392	1.0000	0.365	1.217
Acetaminophen related compound D	0.369–24.611	15230310	703.6019	1.0000	0.123	0.369
Acetaminophen related compound J	0.125–0.499	26382500	-88.31898	0.9999	0.050	0.125
ODAA	0.373–24.850	33323090	665.3543	0.9983	0.124	0.373

3.3.7. Solution stability

Solution stability test was performed by analyzing sample and standard solutions at different periods of time (0, 6, 12, 18 and 24 h) for 24 h. Sample solution of acetaminophen was spiked with impurities of acetaminophen at their limit concentrations and standard solution was prepared as described in Section S.3. (Suppl. Mat.). Peak area results of each compound obtained at five different periods of time for 24 h were assessed for RSD% values (Table S10). The obtained results were evaluated and all RSD% values were found below the accepted limit of 5.0%, demonstrating that the standard and sample solutions prepared within the developed method could show a minimum of 24 h solution stability.

3.4. Stress-testing

Stress-testing of acetaminophen, which was separately conducted for 24 h and 15 days, was performed to determine whether ODAA is a degradation or a synthesis impurity of acetaminophen. The samples of acetominophen subjected to stress-testing under the conditions of acidic hydrolysis, alkaline hydrolysis, oxidation degradation, thermal degradation and photolytic degradation were analyzed by UHPLC in terms of possible degradation products. Results obtained from stress-testing study are given in Table 8.

According to the results obtained (Table 8), acetaminophen was not affected from UV light (254 nm), dry heat (60 °C) and oxidative degradation (3.0% H_2O_2) after 24 h. Acetaminophen was slightly affected from acidic hydrolysis after 24 h where 0.0564% unknown degradation products formed. The most significant degradation after 24 h was observed under alkaline hydrolysis where acetaminophen was degraded to form many unknown impurities with a total amount of 0.3876%. Acetaminophen was not affected from UV light (254 nm) and dry heat (60 °C) after 15 days. The molecule was slightly affected from oxidative degradation by 3.0% H_2O_2 after 15 days where 0.0154% unknown degradation products formed. The most significant degradation after 15 days was observed under acidic and alkaline hydrolysis where 0.7621% and 0.8487% unknown degradation products formed, respectively.

Results obtained from stress-testing showed that none of the forced degradation studies affected ODAA, demonstrating that ODAA is not a

Table 8
Stress-testing results of acetaminophen.

Stress condition	Time (day)	% Assay of the impurities						
		Imp-B*	Imp-C*	Imp-D*	Imp-J*	ODAA	Total degradation impurities	Total impurities
Acidic hydrolysis	0	0.0018	ND	ND	ND	≤ LOQ	–	0.0018
(1.0 N HCl)	1	0.0017	ND	ND	ND	≤ LOQ	0.0564	0.0581
	15	≤ LOQ	ND	ND	ND	≤ LOQ	0.7621	0.7621
Alkaline hydrolysis	0	0.0018	ND	ND	ND	≤ LOQ	–	0.0018
(1.0 N NaOH)	1	0.0030	ND	ND	ND	≤ LOQ	0.3876	0.3906
	15	0.0033	ND	ND	ND	≤ LOQ	0.8487	0.8520
Oxidation degradation	0	0.0018	ND	ND	ND	≤ LOQ	–	0.0018
(3.0% H_2O_2)	1	0.0018	ND	ND	ND	≤ LOQ	≤ LOQ	0.0018
	15	0.0020	ND	ND	ND	≤ LOQ	0.0154	0.0174
Thermal degradation	0	0.0018	ND	ND	ND	≤ LOQ	–	0.0018
(Dry heat, 60 °C ± 2 °C	1	0.0018	ND	ND	ND	≤ LOQ	–	0.0018
	15	0.0020	ND	ND	ND	≤ LOQ	≤ LOQ	0.0020
Photolytic degradation	0	0.0018	ND	ND	ND	≤ LOQ	–	0.0018
(UV light, 254 nm)	1	0.0019	ND	ND	ND	≤ LOQ	≤ LOQ	0.0019
	15	0.0019	ND	ND	ND	≤ LOQ	≤ LOQ	0.0019

* : Imp-B: Acetaminophen related compound B, Imp-C: Acetaminophen related compound C, Imp-D: Acetaminophen related compound D, Imp-J: Acetaminophen related compound J; ND: not detected

degradation impurity but a synthesis impurity of acetaminophen. If *p*-aminophenol contains one of its potential impurities called 4,4′-oxydianiline during the acetylation reaction, both *p*-aminophenol and 4,4′-oxydianiline are acetylated. As a result of acetylation of 4,4′-oxydianiline, ODAA is considered to be formed besides acetaminophen.

3.5. Method application

The proposed method was applied to the analyses of different lots (22208733, 22208734 and APAP-2022) of acetaminophen drug substance. Representative chromatograms obtained from standard solution and different lots of acetaminophen samples are provided in Fig. S8, Fig. S9, Fig. S10 and Fig. S11, respectively. All chromatograms were evaluated by peak areas and the results obtained are given in Table S11, Table S12 and Table S13. The chromatograms showed the existence of acetaminophen related compound B, acetaminophen related compound D and unknown impurities in the acetaminophen samples. Impurity results belonging to the acetaminophen samples with lots 22208733 and APAP-2022 were found below the LOQ values (Table S11 and Table S13). In the acetaminophen sample with lot 22208734, acetaminophen related compound B was found to be 0.002%, which is below the accepted limit of 0.05% as described in the supplementary material (Table S12). Thus, it was demonstrated that the proposed method could be used for the routine quality control of organic impurities of acetaminophen.

4. Conclusion

An impurity molecule of acetaminophen which is not specified in USP 42 was synthesized, characterized, standardized, and a new and optimized LC method including this impurity was developed for organic impurities analysis of acetaminophen. Requirements for the validation study of the developed method were fulfilled according to ICH guideline. The validated method has been proved to be sensitive, selective, specific, precise, linear, accurate and robust. The developed LC method provides a good resolution between acetaminophen and its organic impurities, and could be used for the simultaneous determination of acetaminophen related compound B, acetaminophen related compound C, acetaminophen related compound D, acetaminophen related compound J and ODAA. The optimized method was successfully applied to the analyses of different lots of acetaminophen samples for routine application. Compared to the related HPLC method in USP 42, the new method developed for UHPLC instrument offers a short analysis time and uses less mobile phase (Table 9).

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cemil Caner Arıkan reports administrative support and equipment, drugs, or supplies were provided by Atabay Pharmaceuticals and Fine Chemicals Inc. Cemil Caner Arıkan reports a relationship with Atabay

Table 9
Comparative table of USP-42 monograph vs. developed method for analysis of organic impurities of acetaminophen.

	USP-42 method	Newly developed & validated method
	Organic impurities of acetaminophen	Organic impurities of acetaminophen
Method	HPLC	UHPLC
Impurities	Acetaminophen related compound B, Acetaminophen related compound C, Acetaminophen related compound D, Acetaminophen related compound J	ODAA, Acetaminophen related compound B, Acetaminophen related compound C, Acetaminophen related compound D, Acetaminophen related compound J,
Column	Kromasil C8 (250 × 4.6 mm; 5-μm)	InertSustain C8 (150 × 2.1 mm; 2-μm)
Column temperature	40 °C	40 °C
Elution	Gradient	Gradient
Mobile phase	Solvent A: methanol-water-glacial acetic acid (50:950:1, v/v/v); Solvent B: methanol-water-glacial acetic acid (500:500:1, v/v/v)	Solvent A: methanol-water-glacial acetic acid (50:950:1, v/v/v); Solvent B: methanol-water-glacial acetic acid (500:500:1, v/v/v)
Flow rate	0.9 mL/min	0.2 mL/min
Detection	UV, 254 nm	UV, 254 nm
Run time	73 min	30 min
Injection volume	5 μL	1 μL

Pharmaceuticals and Fine Chemicals Inc. that includes: employment.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.115123.

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