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Article

HPLC Separation of Acetaminophen and its Impurities Using A Mixed-mode Reversed-Phase/Cation Exchange Stationary Phase

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Determination of acetaminophen and its main impurities: 4-nitrophenol, 4′-chloroacetanilide, as well as 4-aminophenol and its degradation products, *p*-benzoquinone and hydroquinone has been developed and validated by a new high-performance liquid chromatography method. Chromatographic separation has been obtained on a Hypersil Duet C18/SCX column, using gradient elution, with a mixture of phosphate buffer (pH = 4.88) and methanol as a mobile phase. Analysis time did not exceed 14.5 min and good resolutions, peak shapes and asymmetries have resulted. The linearity of the method has been tested in the range of 5.0–60 μg/mL for acetaminophen and 0.5–6 μg/mL for the other compounds. The limits of detection and quantification have been also established to be lower than 0.1 μg/mL and 0.5 μg/mL, respectively. The method has been successfully applied for the analysis of commercial acetaminophen preparations.

Introduction

Acetaminophen (N-acetyl-*p*-amino-phenol, AAP), also known as paracetamol, is a widespread antipyretic and analgesic accepted as an effective treatment for the relief of pain and fever in adults and children (1). Organic impurities that may appear in acetaminophen preparations are process-related impurities; their profile is influenced by the choice of synthetic route, the quality of the starting materials, reagents and solvents, the reaction conditions, the work-up and final purification and the design of the process equipment (2). The primary impurity of acetaminophen, 4-aminophenol (4-AP) occurs in acetaminophen pharmaceutical preparations as a consequence of both synthesis and degradation during storage (3, 4). The quantity of 4-AP must be strictly controlled as it is reported to have nephrotoxic and teratogenic effects (3). Under accelerated degradation conditions, 4-AP itself may undergo further degradation, the main products being *p*-benzoquinone (BQ) (5) and hydroquinone (HQ) (6).

A significant number of studies for assaying the concentration of 4-AP in acetaminophen drugs have been reported, the methods used including UV spectrometry (7–9), fluorimetry (4), capillary electrophoresis (10, 11), micellar electrokinetic chromatography (3), flow injection analysis (12), and high performance liquid chromatography (13). Literature reports a small number of studies concerning the determination of other major impurities in AAP formulations (2, 14–16). The British Pharmacopoeia (17) describes a chromatographic method of determining the concentrations of 4-nitrophenol (4-NP), 4′-chloroacetanilide (4-CA) and 4-AP as related substances in acetaminophen, using a C8 stationary phase with a mobile phase containing phosphate buffer and methanol spiked with tetrabutylammonium hydroxide in a 75:25 (v/v) ratio, with UV detection at 245 nm. For this method the time of analysis is reported to be around 30 minutes, as 4-CA elutes at a retention time of 28 minutes. Marín and Barbas (18) reported a high-performance liquid chromatography (HPLC) method that uses a polyethylene glycol column and isocratic elution of an ammonium acetate – acetonitrile mobile phase at a 90:10 (v/v) ratio and MS detection.

Lately, a variety of new columns for HPLC which contain two types of mixed mode-phases, octadecylsilane/strong cation exchanger (C18/SCX) and octadecylsilane/strong anion exchanger (C18/SAX) has been commercially available. Combination of two different phases allows for an increased selectivity and more variables for fine tuning separations (19, 20). Scientific literature regarding the use of columns packed with a C18/SCX mixed-mode phase is fairly limited, with some applications in capillary electrokinetic chromatography (21, 22) and HPLC (20, 23, 24).

However, no studies regarding the separation of acetaminophen and its impurities using a mixed mode column that contains both octadecylsilane and strong cation exchanger phases have been published so far.

The goal of the present study was the separation and determination of the process impurities 4-NP, 4-CA and 4-AP and the degradation products of 4-AP: BQ and HQ in mixtures containing acetaminophen. The last two compounds BQ and HQ were introduced in this study as 4-AP is easily oxidized to them even in normal conditions. The official monograph of acetaminophen (17) presents the separation of only AAP, 4-AP, 4-CA and 4-NP using a C8 stationary phase. Other studies (13, 18) present the separation of AAP, 4-AP, 4-CA, 4-NP and other related impurities too, but none cover the separation of HQ and BQ from the above mentioned compounds.

Even though the monograph of acetaminophen exists in many pharmacopoeias in use, there is no specification on the determination of HQ and BQ, together with acetaminophen and other impurities. Besides, until now, no studies have been reported in scientific literature regarding the separation of the main impurities of acetaminophen and the degradation impurities of 4-AP, which decomposes rapidly at room temperature.

This paper reports the development and validation of a new, simple and reliable HPLC method for the simultaneous determination of process impurities 4-NP, 4-CA and 4-AP and of the degradation products of 4-AP: BQ and HQ in mixtures

containing acetaminophen. Based on the data obtained, the HPLC method has been applied for the analysis of commercial acetaminophen preparations.

Experimental

Chemicals

Acetaminophen (≥99%), 4-aminophenol (≥97%), 4'-chloroacetanilide (≥98%), 4-nitrophenol (≥99%), *p*-benzoquinone (≥98%), hydroquinone (≥99%), potassium dihydrogen phosphate, dipotassium hydrogenphosphate, sodium acetate, glacial acetic acid (>99.7%, d = 1.049 g/mL), phosphoric acid (85%, d = 1.685 g/mL), acetonitrile and methanol, both HPLC grade were purchased from Sigma-Aldrich (Deisenhofer, Germany). Potassium nitrate was purchased from Merck (Darmstadt, Germany). Acetic acid 1 M solution was prepared using glacial acetic acid and water for chromatography.

Acetaminophen tablets produced by Centrofarm S.A. (Bucharest, Romania) were bought from the market. Each tablet contains 500 mg of acetaminophen and the following excipients: maize starch, povidone, silica colloidal anhydrous, sodium lauryl sulphate, talc and magnesium stearate.

Apparatus

Chromatographic analysis was performed on a HPLC Finnagan Surveyor system (Thermo Electron Corporation, Waltham, MA) composed of a quaternary pump, an automatic injector, column thermostat, and temperature-controlled sample trays, an on-line degasser and having a Photodiode Array (PDA) detector with a cell length of 50 mm and a fluorescence (FLD) detector. The control was done by the ChromQuest software.

Ultra pure water (18 MΩ/cm) was produced using an ULTRA CLEAR system (Richfield, USA). The pH of the buffer solutions was measured using a pH/mV-meter Consort P501 (Turnhout - Belgium), provided with a combined pH electrode. UV–vis spectra were recorded on a Jasco V-530 spectrophotometer (Tokyo, Japan), in 10 mm quartz cells. Spectramanager software was used for control. Fluorimetric measurements were made on a Fluorolog-3 system (Horiba Jobin Yvon, NJ) using 10 mm quartz cells. Control was done through the FluorEssence software. Ultraviolet lamp 245/365 nm (Cole-Parmer, Vernon Hills) has been used for the forced degradation studies.

Solutions

Solutions for spectrometric and fluorimetric measurements

Stock solutions of 50 μg/mL of each AAP, 4-AP, 4-CA, 4-NP, BQ and HQ were prepared by dissolving 0.0005 g of the respective substance in HPLC grade methanol in a 10 mL volumetric flask, filled up to the mark with the same solvent.

Working solutions of each compound containing the respective substance at a 5 μg/mL concentration were prepared by dilution of 1 mL stock solution in a 10 mL volumetric flask, using methanol.

Solutions for HPLC

A stock solution of AAP containing 200 μg/mL was prepared in a 10 mL volumetric flask, by dissolving 0.0020 g AAP in methanol (HPLC grade). The flask was filled up to the mark with the same solvent.

Stock solutions 50 μg/mL of 4-AP, 4-CA, 4-NP, BQ and HQ respectively were prepared in a 10 mL volumetric flask by dissolving 0.0005 g of the respective substance in methanol for chromatography. Each flask was filled up to the mark with methanol HPLC grade.

Working solutions containing all the analytes were prepared in the range of 5.0–60 μg/mL AAP and 0.5–6 μg/mL for the impurities using methanol HPLC grade as diluting solvent. All analyte solutions were prepared daily and kept at 4°C before and between injections to prevent sample degradation.

A phosphate buffer solution of pH = 4.88 was prepared by dissolving 4.5 g KH_2PO_4 and 0.0412 g $K_2HPO_4 \cdot 3H_2O$ in 500 mL of ultra pure water. If necessary the pH could be adjusted to pH = 4.88 with phosphoric acid (85%).

An acetate buffer solution of pH = 4.10 was prepared by mixing 0.4512 g CH_3COONa and 32 mL CH_3COOH 1M in a 500 mL flask, filled up to the mark with ultra pure water. A stock solution of potassium nitrate was prepared by dissolving 0.0326 g in water in a 50 mL volumetric flask filled up to the mark with the same solvent.

Analysis of acetaminophen tablets

Three tablets of acetaminophen (Centrofarm S.A., Romania) were accurately weighed and then finely pulverized in a mortar. Out of this quantity 0.3400 g were accurately weighed and then dissolved in methanol in a 25 mL volumetric flask filled up to the mark with the same solvent. This solution was filtered through a 0.45 μm syringe filter and then 5 mL of filtrate were transferred to a 10 mL volumetric flask that was filled up to the mark with methanol. This solution was injected into the chromatographic system.

Forced degradation studies

The effects of two different wavelengths of UV radiation on acetaminophen tablets (Centrofarm S.A., Romania) were assessed as follows: (*i*) Three acetaminophen tablets were accurately weighed and then finely pulverized in a mortar. The resulting powder was irradiated under UVA radiation (365 nm) for 1 h, after that a quantity of 0.3400 g of the powder was accurately weighed and then dissolved in a 25 mL volumetric flask filled up to the mark with methanol. This solution was filtered through a 0.45 μm syringe filter. A volume of 5 mL of filtrate was transferred to a 10 mL volumetric flask and filled up to the mark with methanol. This solution was injected into the chromatographic system.

(*ii*) The resulting filtrate from experiment (*i*) was used to test the effect of temperature on acetaminophen degradation in solution. The methanolic solution of acetaminophen was kept for five hours at 40°C and then injected into the chromatographic system.

(*iii*) The resulting filtrate from experiment (*i*) was kept in laboratory for 60 days at 25°C. A volume of 20 μL of this solution was transferred to a 10 mL volumetric flask which was filled up

to the mark with methanol. This solution was injected into the chromatographic system.

(*iv*) Three tablets of acetaminophen were accurately weighed and then ground to a fine powder in a mortar. The resulting powder was kept in the laboratory at 25°C for 60 days. A quantity of 0.3400 g was accurately weighed and then dissolved in a 25 mL volumetric flask filled up to the mark with methanol. This solution was filtered through a 0.45 μm syringe filter and then injected into the chromatographic system.

(*v*) Three acetaminophen tablets were accurately weighed and then finely ground in a mortar. The resulting powder was put under UVC radiation (245 nm) for 1 hour. After this period a quantity of 0.3400 g was accurately weighed and then dissolved in a 25 mL volumetric flask filled up to the mark with methanol. This solution was filtered through a 0.45 μm syringe filter and then injected into the chromatographic system.

Chromatographic conditions

Chromatographic studies were performed using a Hypersil Duet C18/SCX column, purchased from ThermoElectron corporation, USA, with the dimensions 250 x 4.6 mm and 5 μm particle size. The injection volume was 5 μL. The final separation method used a mobile phase containing phosphate buffer (pH = 4.88)–methanol and the following gradient program: from 0 to 8 min the percent of organic modifier varies from 20% to 50%, remains constant for 2 minutes and increases to 60% for another 2 min. Flow rate was set at 0.8 mL/min for the first 12 min of the separation, then increased to 1.2 mL/min between 12–14 min. Detection was made at 230 nm. Both the sample holder and the column were kept at 25°C.

Validation of the method

The method was validated according to international rules (25, 26).

Software

Theoretical pK_a values were calculated using the ACD/pK_a DB version 6.0 for Microsoft Windows.

Results and Discussion

HPLC method development

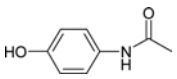
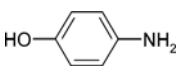
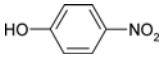
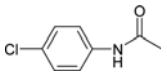
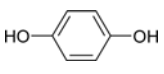
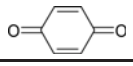
Acetaminophen and all the compounds enclosed in this study ionize according to the pH of the mobile phase. Usually, the separation of organic charged molecules is performed by ion-pair chromatography. Even if it has a lot of advantages, the corrosive action of a many counter-ions on the stationary phase of the column constitutes a practical limitation of ion-pair chromatography.

For this reason, a mixed-mode stationary phase can surpass the limitations of ion-pair chromatography and can be able to separate simultaneously both ionic and neutral organic molecules without any practical restriction.

The development of this new HPLC method is based on the behavior of ionizable compounds that have the retention closely related to the pH of the mobile phase (27). In order to establish the distribution of the conjugated acid/base species as a function of pH for all the analytes that have been studied in this paper, we have computed their pK_a values. The results are presented in Table I.

As shown in Table I, acetaminophen exists in two acid/base conjugated species: in its acid form (–OH) in the pH range (0 – 9.86) and in the conjugated base form ($-O^-$) of this acid at pH values greater than 9.86. 4-Nitrophenol is in its acid form (–OH) at pH values lower than 7.23, and in its conjugated base form ($-O^-$) at pH values greater than 7.23. 4-Aminophenol has two pK_a meaning that this compound exists in three acid/base conjugated forms: at pH values lower than 5.28 the $-NH_3^+$ form prevails, in the pH range of 5.28 – 10.17 there is the neutral form of aminophenol, while at pH values greater than 10.17 its anionic form ($-O^-$) exists. Hydroquinone is ionic ($-O^-$) at pH values greater than 10.33, while benzoquinone and 4-chloroacetanilide are non ionic whatever the pH is between

Table I
Chemical Structures and pK_a Values for Acetaminophen and its Impurities

Compound	Molecular formula	Chemical structure	pK_{a_1}	pK_{a_2}	pK_{a_3}
Acetaminophen	$C_8H_9NO_2$		-0.14 ± 0.50 (NH_2^+/NH)	9.86 ± 0.13 (OH/O^-)	15.32 ± 0.70 (NH/N)
4-Aminophenol	C_6H_7NO		5.28 ± 0.10 (NH_3^+/NH_2)	10.17 ± 0.13 (OH/O^-)	–
4-Nitrophenol	$C_6H_5NO_3$		7.23 ± 0.13 (OH/O^-)	–	–
4′-Chloroacetanilide	C_8H_8ClNO		-1.97 ± 0.50 (NH_2^+/NH)	14.25 ± 0.70 (NH/N)	–
Hydroquinone	$C_6H_6O_2$		10.33 ± 0.13	11.86 ± 0.13	–
p-Benzoquinone	$C_6H_4O_2$		–	–	–

0 and 14. Since BQ, HQ, 4-AP and AAP have similar structures, their separation as non-ionic compounds may be problematic. However, since the Duet C18/SCX column is capable of separating both non-ionic and cationic species, a pH at which one of these four components is a cation was preferred. At a pH between 4 and 5, five compounds: AAP, 4-NP, 4-CA, HQ and BQ are in a non-ionized form, while 4-AP is in a cationic one.

Initial determinations were made using an aqueous acetate buffer (pH = 4.10). The elution was isocratic using a buffer – methanol (80:20 v/v) mobile phase. First, chromatograms were separately traced for the six compounds. The retention times under these conditions for 4-AP, HQ, AAP, BQ, 4-NP, 4-CA were 4.20, 4.27, 4.95, 5.58, 15.16 and 38.12 min respectively. Recording a chromatogram of a mixture containing the six analytes proved that 4-AP and HQ co-elute under the above working conditions. Subsequent attempts were made at higher buffer contents of the mobile phase, but a mobile phase of buffer – methanol up to 92:8 (v/v) was still unable to separate the two analytes.

Therefore, further attempts of separation were made using a different buffer solution, while still maintaining a pH at which 4-AP would be in its protonated form. A potassiumdihydrogen phosphate/dipotassiumhydrogen phosphate buffer at a pH of 4.88 was chosen for further experiments. First chromatographic experiments under isocratic conditions made using a mobile phase buffer (pH = 4,88)–methanol (80:20 v/v) showed good separation between the analytes, with retention times for HQ, AAP, BQ, 4-AP, 4-NP and 4-CA of 4.47, 5.34, 5.94, 6.51, 18.8 and 49.9 min, respectively, and chromatographic resolutions of 3.26, 2.24, 2.15, 24.05 and 22.65 respectively. Since the total analysis time was quite high (a single run took 52 min) further experiments were developed using gradient elution to decrease the total time of analysis by reducing the retention of 4-NP and especially of 4-CA. The separation was optimized to the gradient elution and the flow rate gradient program described in the experimental section. Under these conditions the time of analysis is under 15 min and the selectivity and the efficiency of the separation remain high.

Under these optimal chromatographic conditions, the elution times for HQ, AAP, BQ, 4-AP, 4-NP and 4-CA were 4.45, 5.29, 5.89, 6.99, 11.52, and 14.07 min, respectively, with resolutions of 3.65, 2.53, 4.27, 17.67, and 13.31, respectively. A sample chromatogram of a working solution of a mixture of the six analytes is presented in Figure 1. All peaks have good symmetry, with asymmetry values between 0.85 and 1.15. A baseline drift usually associated with gradient elution exists, but is very low.

Fluorescence spectra were also recorded for all analytes, as fluorescent detection is known to be highly sensitive and offers lower detection limits. All compounds are detectable by fluorescence except 4-NP which is known to be a non-fluorescent compound (28). Based on the individual fluorescence spectra of each analyte the wavelengths of excitation and emission for them were chosen and given in Table II.

A fluorescence program for HPLC detection was also developed, with the aim of analysing samples with low concentrations of acetaminophen impurities. Therefore, the excitation wavelength was chosen to be one at which acetaminophen does not manifest high fluorescence. The proposed program has a fixed excitation wavelength of 247 nm, with a fixed emission wavelength of 356 nm. Under these conditions all compounds except 4-NP can be detected with high sensitivity.

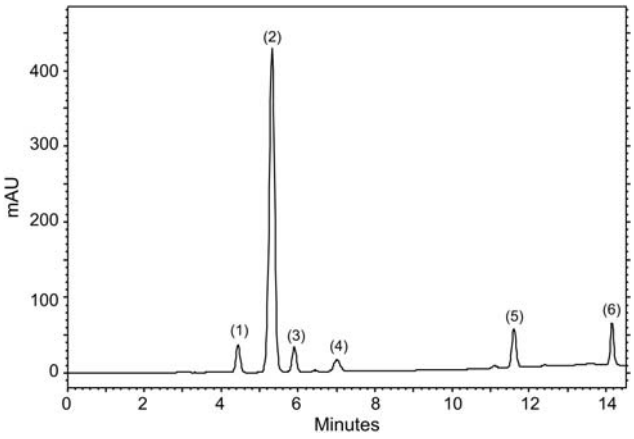


Figure 1. Sample chromatogram of a reference solution containing the six analytes using a mobile phase of phosphate buffer–methanol 80:20 (v/v) and gradient elution. Peak 1, hydroquinone; Peak 2, acetaminophen; Peak 3, *p*-benzoquinone; Peak 4, 4-aminophenol; 5, 4-nitrophenol; 6, 4′-chloroacetanilide.

Table II
Excitation and Emission Wavelengths Used for Fluorescence Detection

Compound	Excitation wavelength (nm)	Emission wavelength (nm)
Acetaminophen	290	355
4-Aminophenol	247	368
4′-Chloroacetanilide	247	363
Hydroquinone	247	356
p-Benzoquinone	247	354

Influence of the organic modifier in the mobile phase

In order to assess the influence of the organic modifier the developed method was modified to use acetonitrile instead of methanol in the mobile phase, while maintaining the same elution conditions as described in the experimental section. Retention times were 3.58 min for HQ, 4.18 min for AAP, 5.28 min for 4-AP, 6.54 min for BQ, 9.15 min for 4-NP and 9.94 min for 4-CA. As expected, the use of acetonitrile decreased the total analysis time, by lowering the retention of 4-NP and 4-CA. However, while the total analysis time was reduced, the symmetry and resolution of the HQ peak was visibly diminished. Moreover, due to the low solubility of the inorganic salts from the buffer solution in acetonitrile potential problems may occur during the gradient modification of the mobile phase composition. A major undesired effect might be the precipitation of phosphate salts, especially when the ratio of buffer – acetonitrile is too low.

Thermodynamic study

From the thermodynamical point of view, the distribution coefficient $K = \exp(-\Delta G/RT)$ is related to the change of the Gibbs energy, G of the compound when it is transferred from the mobile into the stationary phase. The distribution coefficient is related to the corresponding enthalpy and entropy changes (29). Under chromatographic conditions, the temperature

dependence of the retention factor k' can be described by the van't Hoff equation (30, 31, 32):

$$\ln k' = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R} + \ln \varphi \qquad (1)$$

where k' is the retention factor and φ the phase ratio of the column.

The chromatographic behavior of the studied compounds may be appreciated on the basis of the value of the retention factor, k'. The retention factor may be calculated even in the case of gradient elution (33), as is the case of the presently developed method.

The plot of $\ln k'$ versus 1/T has a slope of - $\Delta H^{\circ}/R$ and an intercept of $\Delta S^{\circ}/R + \ln\varphi$. Therefore, the enthalpy of the solute partitioning can be calculated from the slope, and the entropy term can be determined from the intercept.

Capacity factors for all analytes were determined by injecting a solution containing all six analytes under the chromatographic conditions described in the Experimental section, and changing the temperature in the range of 25 – 55°C. The dead time was determined by the injection of a potassium nitrate solution (1000 μg/mL). The plots of $\ln k'$ versus 1/T show a linear dependence for all the analytes with the following equations: $y = 1593.83x - 6.25$ ($R^2 = 0.9993$) for HQ, $y = 1849.00x - 6.60$ ($R^2 = 0.9992$) for AAP, $y = 1200.09x - 4.17$ ($R^2 = 0.9978$) for BQ, $y = 1197.13x - 3.82$ ($R^2 = 0.9885$) for 4-AP, $y = 1356.52x - 3.57$ ($R^2 = 0.9957$) for 4-NP and $y = 789.85x - 1.41$ ($R^2 = 0.9918$) for 4-CA.

Consequently, the enthalpy values determined (kJ·mol^{-1}) were of −9.98, −13.25, −15.37, -9.95, -11.28 and -6.57 for HQ, AAP, BQ, 4-AP, 4-NP and 4-CA, respectively. Figure 2 presents the plots of ln k' versus 1/T. The linear character of the mentioned plots also shows that the thermodynamic parameters are temperature independent in the experimental range of 25 – 55°C. The linearity of the van't Hoff plots indicates that the retention mechanism of each compound does not change when temperature increases.

Only for 4-AP the variation of ln k' intersects the 1/T axis, at 40 °C. At the pH value of the aqueous component of the mobile phase (4.88), 4-AP mostly exists in a cationic form. This behavior of 4-AP outlines the advantage of using a mixed mode C18/SCX stationary phase under favorable thermodynamic conditions and the possibility of controlling separation processes through the variation of the mobile phase pH. This fact is a great advantage of mixed mode stationary phases compared to those exclusively non-polar or ion-exchangers.

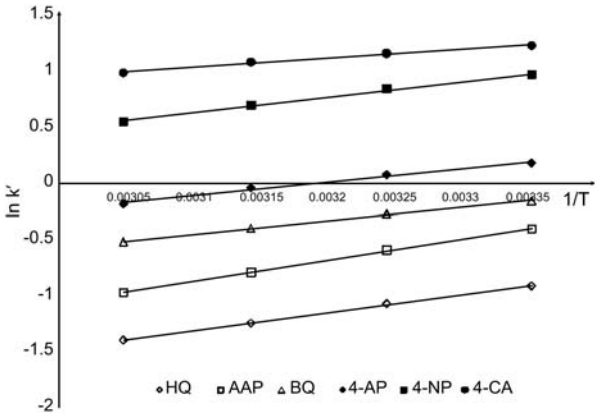


Figure 2. Variations of ln $k' = f(1/T)$ for all the studied compounds.

The solute transfer between the mobile and stationary phases is highly complex, and cannot be elucidated only on the basis of the thermodynamic parameters (34).

Method validation

The final developed method (as described in the Experimental section) was validated, checking for linearity, limits of detection and quantification, accuracy and precision.

Linearity

Linearity has been checked directly for each compound studied in this paper by dilution of a standard stock solution (26). The slope and y-intercept were provided as equation together with the correlation coefficient in order to demonstrate the linearity of the developed method.

Linearity was established for working solutions containing concentrations between 5.0 – 60 μg/mL for acetaminophen and concentrations of 0.5 – 6 μg/mL for the impurities, analogous to a highly degraded acetaminophen sample. The equations obtained are: $y = 56740x + 1243$ ($R^2 = 0.9999$) for HQ, $y = 84431x + 77536$ ($R^2 = 0.9998$) for AAP, $y = 52373x + 16851$ ($R^2 = 0.9922$) for BQ, $y = 38125x + 3166$ ($R^2 = 0.9994$) for 4-AP, $y = 81855x + 4819$ ($R^2 = 0.9956$) for 4-NP and $y = 70578x + 3572$ ($R^2 = 0.9995$) for 4-CA.

Limits of detection and quantification

Limits of detection (LOD) were calculated as the concentration of the analyte which gave a peak 3 times higher than the baseline noise of a methanol injection. The LODs found are: 0.08 μg/mL for HQ, 0.03 μg/mL for AAP, 0.03 μg/mL for BQ, 0.1 μg/mL for 4-AP, 0.045 μg/mL for 4-NP and 0.017 μg/mL for 4-CA.

Limits of quantification (LOQ) were calculated as the concentration of the analyte which gave a peak 10 times higher than the baseline noise of a methanol injection. The LOQs determined are: 0.25 μg/mL for HQ, 0.1 μg/mL for AAP, 0.1 μg/mL for BQ, 0.46 μg/mL for 4-AP, 0.14 μg/mL for 4-NP and 0.12 μg/mL for 4-CA.

Accuracy

Accuracy was determined by calculating the recovery of each analyte from synthetic samples corresponding to three concentration levels ranging from 50 ± 20% μg/mL AAP and 5 ± 20% μg/mL of each impurity. The obtained values for the recoveries were in the range 99.6 – 102.8%. The results are presented in Table III.

Precision

The precision was estimated by both repeatability and intermediate precision. According to the international rules of validation (26) repeatability was checked on six identical solutions containing 50 μg/mL AAP and 5 μg/mL impurities (representing 100% of the test concentration) and on two other concentrations covering the range of 80% and 120% of the test

Table III
Accuracy and Precision of the Developed Method

Compound	Concentration	Recovery [% ± s.d.]	Precission (% R.S.D.)	
			Intra-day	Inter-day
Acetaminophen, AAP	40 μg/mL	100.7 ± 0.42	1.29	1.45
	50 μg/mL	99.90 ± 0.03	0.33	0.27
	60 μg/mL	101.2 ± 0.87	0.78	0.87
4-Aminophenol, 4-AP	4 μg/mL	100.09 ± 0.94	1.78	1.08
	5 μg/mL	99.86 ± 1.27	1.87	1.33
	6 μg/mL	98.71 ± 1.84	1.49	0.83
4-Nitrophenol, 4-NP	4 μg/mL	98.49 ± 1.33	1.29	1.87
	5 μg/mL	102.8 ± 1.96	0.37	1.78
	6 μg/mL	100.2 ± 1.07	0.78	1.96
4′-Chloroacetanilide,	4 μg/mL	99.51 ± 1.72	1.08	0.39
	5 μg/mL	100.9 ± 0.56	0.77	0.54
	6 μg/mL	98.27 ± 0.64	0.89	0.41
Hydroquinone, HQ	4 μg/mL	101.56 ± 1.78	1.67	0.94
	5 μg/mL	99.62 ± 1.63	1.61	1.89
	6 μg/mL	98.07 ± 1.91	1.54	1.14
p-Benzoquinone, BQ	4 μg/mL	99.24 ± 1.82	1.35	0.97
	5 μg/mL	100.24 ± 1.47	1.96	1.74
	6 μg/mL	101.32 ± 1.17	1.58	1.37

Note: s.d. = standard deviation ; R.S.D. = relative standard deviation.

concentration. Intermediate precision was established on another day, by a different investigator using.

Precision was assessed by calculating the relative standard deviation (RSD). The obtained values for the RSD. are below 2% showing a good precision of the method. The results are presented in Table III.

The reproducibility was checked on aliquots of acetaminophen tablets with different analysts. Reproducibility was expressed in terms of relative standard deviation, R.S.D. The values obtained for R.S.D. (%) were: 0.17%, 2.14%, 1.89%, 1.26%, 1.78% and 0.96% for AAP, HQ, BQ, 4-AP, 4-NP and 4-CA respectively. There results show a good reproducibility of the HPLC method.

Analysis of acetaminophen preparations

Commercially available acetaminophen tablets (Centrofarm S.A., Romania) reported to contain only acetaminophen and excipients were bought from the pharmacy and analysed using the validated chromatographic method as described in the experimental section. Results show the existence of 4-AP in the sample solution as well as that of a second, unidentified impurity, with a retention time of 9.3 min. Concentration of 4-AP was determined to be 0.238 mg 4-AP/g of tablet, corresponding to 0.032 parts of 4-AP to 100 parts of AAP; this value is under the accepted limit as described by the British Pharmacopoeia (17).

Analysis of acetaminophen tablets irradiated with UVA light (365 nm) revealed no increase in the concentration of 4-AP, as is to be expected due to the low absorption of AAP in this region of the UV spectrum (35). Analysis of the same solution subjected to high temperature, however, gave an increase in 4-AP concentrations up to 0.279 mg 4-AP/g of tablet corresponding to 0.037 parts of 4-AP to 100 parts of AAP.

Analysis of the solution subjected to 25°C for 60 days showed severe degradation of AAP (Figure 3). The concentration of 4-AP was determined as 93.22 mg/g of tablet. The concentration of AAP decreased to 248.97 mg/g of tablet from a declared one of 744.38 mg/g of tablet. This gives a ratio of

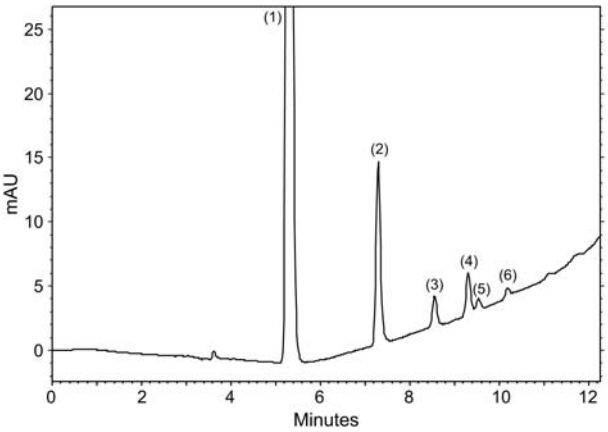


Figure 3. Sample chromatogram of a methanolic solution of acetaminophen prepared as described in section Forced degradation studies (iii) kept at 25°C for 60 days: Peak 1, acetaminophen; Peak 2, *p*-aminophenol; Peaks 3, 4, 5, and 6 unknown compounds.

37.46 parts of 4-AP to 100 parts of AAP. In addition to the peaks corresponding to AAP and 4-AP a further number of four significant peaks were identified with retention times of 8.55, 9.31, 9.54 and 10.19 min, respectively. The resolution between 4-AP and the unknown peak that elutes at 8.55 min is 6.88, while the resolution between the peaks located at 9.31 and 9.54 is 1.23. These four unknown degradation compounds could be identified in this method only based on their UV–vis absorption spectra recorded by the PDA detection system. The structure of these four compounds was not elucidated.

Acetaminophen tablets kept at 25°C for 60 days showed much lower degradation than the solution prepared as described in section 2.5. b) . The concentration of 4-AP obtained was 0.303 mg/g of tablet, or 0.041 parts of 4-AP to 100 parts of AAP.

Acetaminophen tablets irradiated by UVC light (254 nm) showed an increase of the concentration of 4-AP compared to the undegraded tablets. Thus, the determined concentration of 4-AP was of 0.274 mg/g of tablet, or 0.037 parts of 4-AP to 100 parts of AAP. The chromatogram also revealed an increase in the peak area corresponding to the unknown impurity with a retention time of 9.31 min compared to the undegraded tablets. In addition, it was shown that HQ was also present in the sample at a concentration, which corresponds to 0.035 mg HQ/g of tablet, or 0.0047 parts of HQ to 100 parts of AAP.

Conclusions

A new HPLC method for the determination of acetaminophen and its impurities: 4-aminophenol, acetaminophen, 4-nitrophenol, *p*-benzoquinone, hydroquinone, 4'-chloroacetanilide was developed using a mixed-mode Hypersil Duet C18/SCX stationary phase. The separation was achieved using a mixture of phosphate buffer (pH = 4.88)–methanol (80:20 v/v) as mobile phase. Both elution and flow rate gradients contribute to the total time of analysis lower than 15 minutes. The selectivity and the efficiency of the separation are very good. The chromatographic method was validated in the laboratory. A fluorescence program for detection in HPLC was also developed, with

the aim of analyzing samples with low concentrations of acetaminophen impurities. The influence of the temperature on the separation of the analyte was also studied; the linearity of the van't Hoff plots indicates that the retention mechanism of each compound does not change when temperature increases. The method was applied with good results on commercially available acetaminophen tablets.

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