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A validated, sensitive HPLC method for the determination of trace impurities in acetaminophen drug substance

Marika Kamberi*, Christopher M. Riley, Xiaoyan Ma (Sharon), Chen-Wen C. Huang

Department of Analytical Sciences, ALZA Corporation, Mountain View, 1501 California Avenue, Palo Alto, CA 94304, USA

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Abstract

A high-performance liquid chromatography (HPLC) method has been developed and validated for the simultaneous determination of *n*-propionyl-*p*-aminophenol, 3-chloro-4-hydroxyacetanilide, 4′-hydroxyacetophenone, 4-hydroxyacetophenone oxime, 4-acetoxyacetanilide and 4′-chloroacetanilide, the main impurities in acetaminophen drug substance. The chromatographic separation was achieved on an Eclipse XDB-18 reversed-phase column using a gradient elution, being solvent A: 0.01 M phosphate buffer at pH 3.0 and solvent B: methanol. The limit of quantitation ($S/N = 10{:}1$) was 0.1 μg/ml for each impurity. The coefficients of variation were less than 4% for intra-day and inter-day analyses. The individual recovery of acetaminophen spiked samples ranged from 94 to 104% and the mean recovery for each level from 99 to 103% in the 1–150 μg/ml range for all impurities. The proposed method was successfully applied to the analyses of different lots and different manufactures of acetaminophen drug substance. The proposed method can be used for the routine quality control of acetaminophen.

Keywords: HPLC; Acetaminophen; Impurities; Drug substance

1. Introduction

Acetaminophen (*n*-acetyl-*p*-aminophenol, APAP) is a valuable non-steroidal anti-inflammatory drug in widespread use for the management of pain and fever and in a variety of patients, including children, pregnant women, the elderly; and those with ostheoartheritis, simple headaches, and non-inflammatory musculoskeletal conditions [1,2].

Organic impurities [*n*-propionyl-*p*-aminophenol (P-PAP), 3-chloro-4-hydroxyacetanilide (C-APAP), 4′-hydroxyacetophenone (4-HAP), 4-hydroxyacetophenone oxime (4-HAP Oxime), 4′-chloroacetanilide (4-CAA) and 4-acetoxyacetanilide (4-AAA)] (Fig. 1) are process-related impurities that may be present in acetaminophen drug substance.

Their profiles are influenced by the choice of synthetic route; the quality of starting materials, reagents and solvents; the reaction conditions; the work-up and final purification; and the design of process equipment. Since impurities can have safety and efficacy implications, they are the subject of considerable attention by both the manufacturer and regulatory agencies [3–5]. Thus, an analytical method to go hand-in-hand with process design is needed to detect and identify these impurities. In addition, the importance of qualifying impurity profiles is also relevant to the development

* Corresponding author. Tel.: +1-650-564-2217; fax: +1-650-564-2074.
E-mail addresses: marika.kamberi@alza.com, kmarika55@hotmail.com (M. Kamberi).

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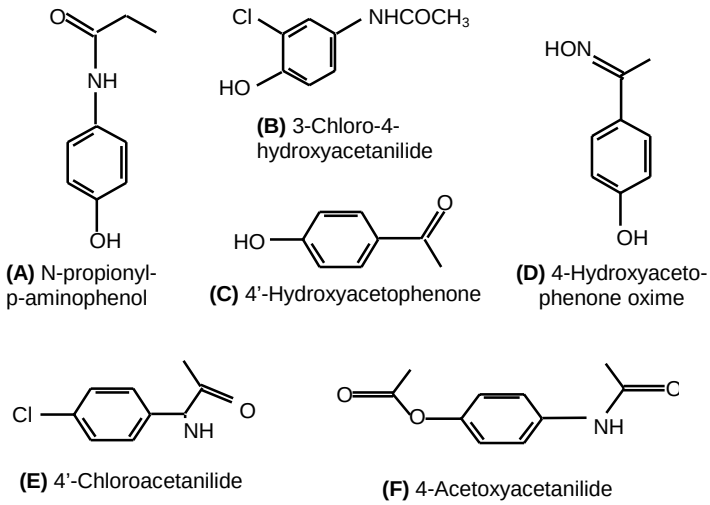

Fig. 1. Chemical structures of six acetaminophen impurities.

scientists to ensure consideration is given to the impurities present in batches being used in safety studies.

So far, the present compendial monographs (United States Pharmacopeia, European Pharmacopeia, Japanese Pharmacopeia, or British Pharmacopeia) for acetaminophen do not include HPLC methods for the potential impurities most likely to arise during the syntheses and which can contribute to the impurity profile of the drug substance. Two recent articles [6,7] have described reversed-phase (RP)-HPLC determination of only 4-aminophenol and 4-chloroacetanilide in pharmaceutical preparations.

The purpose of this work was to develop and validate a highly sensitive, specific and reproducible method for determination of several impurities that may be present in acetaminophen drug substance in a single chromatographic run. This could potentially improve the efficiency of the analysis and reduce laboratory supply costs associated with revalidating and testing of methods for individual impurities.

2. Experimental

2.1. Instrumentation and chromatography

Chromatography was performed with an Agilent 1100 series HPLC system (Agilent Technologies, Inc., California, USA) provided with a binary pump, a thermostatted autosampler, a thermostatted column compartment, and a multiple wavelength diode array detector (DAD)/ultraviolet (UV) detector. Data were collected and analyzed using Turbochrom Client Server Software, version 6.2 (Perkin-Elmer, Inc.). The separation of analytes was accomplished using an Eclipse XDB-C18 reversed-phase column (4.6 mm i.d. × 150 mm, 3.5 μm) (Agilent Technologies, Inc.) maintained at 50 °C. Final chromatographic conditions involved a gradient elution, with solvent A: 0.01 M phosphate buffer solution (sodium phosphate dibasic Na_2HPO_4), adjusted to pH 3.0 with phosphoric acid and solvent B: methanol. The pump flow rate was 1 ml/min. The mobile phase program is described in Table 1.

Table 1
Mobile phase program for gradient elution

Time (min)	Flow (ml/min)	Solvent A (%)	Solvent B (%)	Curve
0	1.0	94	6	Linear
15	1.0	85	15	Linear
20	1.0	70	30	Linear
30	1.0	27	73	Linear
31	1.0	6	94	Linear
35	1.0	6	94	Isocratic
36	1.0	94	6	Linear
45	1.0	94	6	Isocratic

Solvent A: 0.01 M phosphate buffer at pH 3, solvent B: methanol.

The injection volume was 5 μl. The optimum wavelength of 254 nm, which represents the wavelength of maximum absorbancy of all six impurities, was selected in order to permit their simultaneous determination in acetaminophen drug substance.

2.2. Chemicals

All chemicals were at least analytical grade. HPLC grade methanol and phosphoric acid were obtained from JT Baker (New Jersey, USA). Na_2HPO_4 was purchased from Mallinckrodt Baker, Inc. (Paris, France). HPLC grade water was prepared by purifying demineralized water in a Milli-Q filtration system (Millipore, Bedford, MA). Reference standards for 4-HAP Oxime, 4-AAA, and P-PAP were kindly supplied by BASF Co. (Texas, USA). The reference standard for C-APAP was obtained from Lancaster Syntheses Inc. (Windham, USA). 4-HAP and 4-CAA were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Different lots of acetaminophen raw material were obtained from BASF Co. and Rhodia (Boulogne, France).

2.3. Standard solutions and sample preparation

A stock solution of six impurities was prepared in a 50 ml volumetric flask with 100 mg of each impurity weighed exactly and dissolved with methanol. The mixture was sonicated for 10 min and adjusted to the volume with methanol. The working standards (1–200 μg/ml) for each impurity were freshly prepared from the stock solution by serial dilution with methanol. For preparation of acetaminophen samples, 1 g of drug substance was weighed accurately into a 100 ml volumetric flask, dissolved with methanol by sonication and adjusted to the volume. An aliquot of the sample was filtered with a 0.45 μm Acrodisk (LC 13 PVDF Gelman) to the vials for injection.

3. Results and discussion

3.1. Specificity

Representative chromatograms obtained by the described method are shown in Fig. 2. The retention

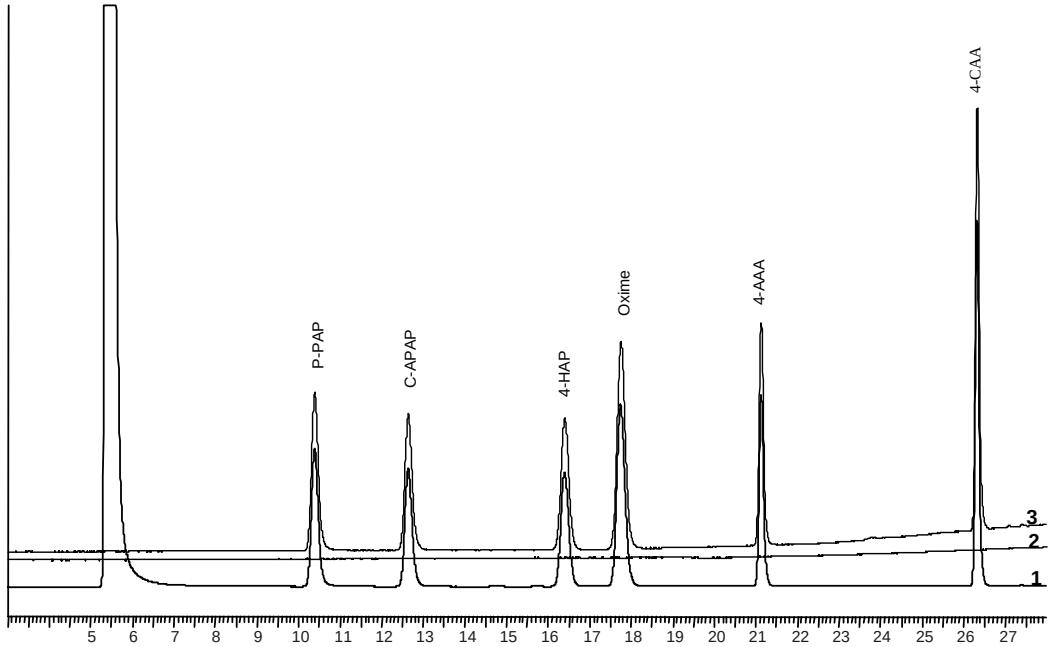

Fig. 2. Representative chromatograms of (1) spiked acetaminophen drug substance sample, (2) blank (mobile phase), and (3) impurities' standard (5 μg/ml).

time for P-PAP, C-APAP, 4-HAP, 4-HAP Oxime, 4-AAA, and 4-CAA was 10.4, 12.7, 16.4, 17.8, 21.1, and 26.3 min, respectively.

Validation of the method was conducted in two phases: (a) the analytical development phase in which the method conditions were defined, and (b) application to the actual analyses of samples from different lots and different manufactures of acetaminophen raw material. The specificity was evaluated by individual injection of six reference standards, the blank mobile phase, the working standard solution mixture, and unspiked and spiked acetaminophen raw material samples. In regard to specificity, there was evidence that the substances being quantitated were the intended analytes. No interference was observed at the same or at ±5% of retention time of each known impurity when the analytes were individually analyzed, and all of the impurities were well resolved from the drug. In addition, some other unknown compounds present in the samples of drug substance were also resolved (Fig. 3).

The concentration range over which six impurities can be determined (1–200 μg/ml, which is equivalent to 0.01–2% of drug substance) was defined based on the evaluation of actual standard samples over the range, including their statistical variation. Seven standards were used to define adequately the relationship between concentration and response. The relationship between response and concentration was demonstrated to be linear and reproducible. The calibration curves were obtained by least-squares linear fitting of the impurity peak areas versus the respective impurity concentrations. The sum of squares for residuals was equal to zero and their scatter (standard deviation) was the same, which indicates that the calibration lines for each impurity were described by a linear relationship. The correlation coefficients between peak area and concentration for each impurity were >0.999. A calibration curve was generated for each analytical run and was used to calculate the concentrations of each impurity in unknown samples assayed with that analytical run.

The limits of detection, based on a signal-to-noise ratio of 3:1, were 0.05 μg/ml for P-PAP, C-APAP, 4-HAP and 4-HAP Oxime, 0.025 μg/ml for 4-AAA, and 0.01 μg/ml for 4-CAA. The limits of quantitation as determined by precision and accuracy and where

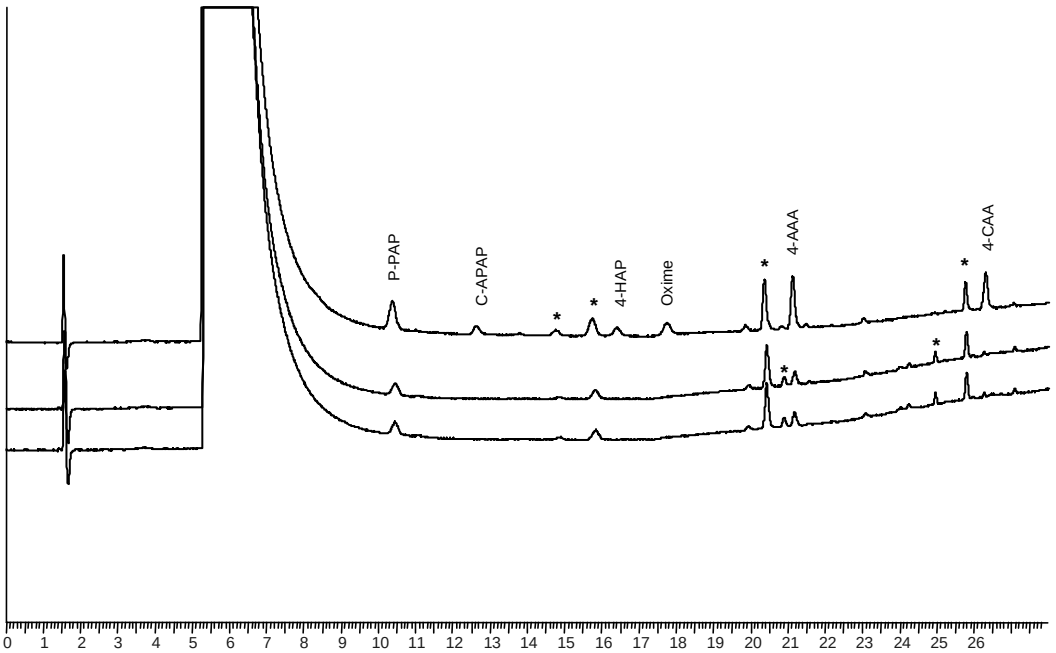

Fig. 3. Representative chromatograms from the analyses of commercially available acetaminophen drug substance from different lots and different manufactures.

the coefficient of variation of <10% were targeted were 0.12 μg/ml for P-PAP, C-APAP, 4-HAP, 4-HAP Oxime and 4-AAA, and 0.10 μg/ml for 4-CAA.

The accuracy and precision with which known concentrations of each impurity in raw material can be determined were evaluated. The recovery assessment was performed by analyses of acetaminophen samples spiked with known amounts of each impurity at four concentrations representing the entire range of the calibration curve. Mean average recoveries in percent and the percent relative standard deviations (%R.S.D.) for each impurity were 106.7 (%R.S.D. 3.1) for P-PAP, 101.6 (2.5) for C-APAP, 101.4 (2.0) for 4-HAP, 101.4 (2.1) for 4-HAP Oxime, 99.1 (2.4) for 4-CAA and 99.5 (2.2) for 4-AAA (Tables 2 and 3).

The inter-day precision for the impurities was determined by calculating the recoveries of six spiked samples for each compound at the 0.2% level (Table 4).

Table 2
Accuracy of the method

Impurity	Spiked value (μg/ml)	Mean recovery [%R.S.D.][a]
P-PAP	1.0	101.7 [6.7]
	6.3	100.5 [1.0]
	52.0	101.7 [0.2]
	142.0	99.5 [0.5]
C-APAP	1.0	102.7 [5.0]
	6.5	102.5 [1.3]
	55.0	100.1 [0.2]
	145.0	101.3 [0.3]
4-HAP	1.0	102.7 [4.1]
	5.7	101.0 [0.5]
	47.0	101.4 [0.2]
	127.0	100.5 [0.7]
4-HAP Oxime	1.0	102.3 [4.5]
	6.7	100.6 [0.6]
	55.0	101.8 [0.2]
	150.0	100.9 [0.5]
4-CAA	1.0	99.7 [5.2]
	5.5	97.9 [0.9]
	45.0	99.6 [0.1]
	120.0	99.1 [0.3]
4-AAA	1.0	97.7 [3.3]
	6.3	98.5 [0.7]
	51.0	101.7 [0.2]
	137.0	100.2 [0.7]

[a] Mean values represent five spiked samples for each concentration.

Table 3
Intra-day precision of the method

Impurity	Mean recovery [%R.S.D.][a]
P-PAP	106.7 [3.1]
C-APAP	101.6 [2.5]
4-HAP	101.4 [2.0]
4-HAP Oxime	101.4 [2.1]
4-CAA	99.1 [2.4]
4-AAA	99.5 [2.2]

[a] Mean values represent 20 spiked samples (five samples at four concentrations) analyzed on the same day.

System suitability was evaluated by injecting daily the middle level of standard solutions ($n = 5$). The system was deemed to be suitable if the number of theoretical plates for each peak was not less than 30,000 plates, the tailing (peak asymmetry) factor was less than 1.3, the coefficient of variation for the area response was not more than 2%, and the resolution between all adjacent peaks more than 2.

The robustness of the method was evaluated by deliberate variation in the method parameters, such as pH (±0.2 units), column temperature (±5 °C) and flow rate (±10%). The changes in the chromatographic results of the system suitability were monitored by varying these parameters, and it was found that there were no changes in the resolution, selectivity, peak width, or symmetry. Only slight changes in the retention times of all peaks were noted with alteration of the column temperature.

To demonstrate the applicability of the developed method in pharmaceutical analysis without interference problems, the method was applied to the analyses of commercially available acetaminophen drug substance from different lots and different manufactures. Representative chromatograms are provided in Fig. 3.

In conclusion, the HPLC method described here is linear, reproducible, sensitive, selective, and robust, and can be used to separate simultaneously six known and four unknown impurities in acetaminophen drug substance. Further work is being carried out to determine whether the method can be used or adopted for the detection and quantification of these impurities in acetaminophen formulations.

Table 4
Inter-day precision of the method

Impurity	Spiked concentration (μg/ml)	Mean recovery [%R.S.D.][a]/day		
		1	2	3
P-PAP	59.0	100.5 [1.2]	102.9 [2.3]	100.1 [0.6]
C-APAP	60.0	99.4 [1.2]	99.7 [0.4]	99.6 [1.0]
4-HAP	57.0	100.6 [0.8]	101.9 [2.3]	100.4 [1.0]
4-HAP Oxime	61.0	99.7 [1.4]	103.6 [0.6]	101.3 [0.1]
4-CAA	60.0	100.8 [0.3]	102.8 [0.5]	99.9 [0.7]
4-AAA	56.0	99.7 [1.0]	99.9 [2.3]	100.1 [0.4]

[a] Mean values represent five spiked samples for each concentration.

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