Published in final edited form as:

Sep Sci Plus. 2020 January ; 3(1-2): 4–11. doi:10.1002/sscp.201900084.

UHPLC assay and impurity methods for diphenhydramine and phenylephrine hydrochloride oral solution

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Abstract

Many over-the-counter drug products lack official compendial analytical methods. As a result, the United States Pharmacopeia and the United States Food and Drug Administration are seeking to develop and validate new methods to establish analysis standards for the assessment of the pharmaceutical quality of over-the-counter drug products. Diphenhydramine and phenylephrine hydrochloride oral solution, a combination drug product, was identified as needing a compendial standard. Therefore, an ultra-high-performance liquid chromatography method was developed to separate and quantify the two drug compounds and eleven related organic impurities. As part of a robustness study, the separation was demonstrated using different high-performance liquid chromatography systems and columns from different manufacturers, and showed little dependence with changes in flow rate, column temperature, detection wavelength, injection volume and mobile phase gradient. The method was then validated conformant with the International Council for Harmonisation guidelines. For impurities, adequate specificity, linearity, accuracy and precision were demonstrated. For assay, a slight modification to the injection volume was necessary to achieve adequate analytical performance. With successful development and validation, these methods were shown to be suitable for their intended purpose and may be considered for adoption as compendial procedures.

Keywords

diphenhydramine; drug analysis; excipients; phenylephrine; validation

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

1 | INTRODUCTION

The U.S. Food and Drug Administration (FDA) and the United States Pharmacopeia (USP) have both identified several over-the-counter (OTC) drug products in need of modern analysis methods to ensure pharmaceutical quality [1]. Included in this list is diphenhydramine and phenylephrine hydrochloride oral solution, which currently has no U.S. compendial documentary standard despite its widespread availability. Diphenhydramine and phenylephrine hydrochloride oral solution is a combination drug product containing the two drug substances in a single dosage form. While combination products provide advantages (e.g., greater patient compliance in terms of taking the products as advised), the presence of multiple components can complicate testing methodologies because additional substances may interfere with the detection of certain components [2]. To address this, several previous studies have sought to develop a single analysis method capable of determining concentrations of multiple drug substances in oral solutions [3-8]. However, all analysis methods developed to date for diphenhydramine and phenylephrine are only suitable for the assay determination. To our knowledge, there is no report describing a single method to analyze impurities from diphenhydramine and phenylephrine hydrochloride. In addition to the increased number of compounds, the presence of multiple chemically related impurities is more difficult to detect at levels as low as 0.2% (w/w) compared to the drug substances. Further, complicating the separation and detection of impurities are multiple excipients, including inactive substances such as flavorings, dyes and preservatives. While excipients can be found in nearly all drug products, they are especially abundant in oral solution formulations and can complicate analyses of this class of products [9-11].

Therefore, in the work described here, ultra-high-performance liquid chromatography (UHPLC) methods were evaluated for the impurity and assay determinations of diphenhydramine and phenylephrine hydrochloride oral solutions. Methods were tested for robustness and were validated in accordance by International Council for Harmonisation (ICH) Q2(R1) guidelines [12].

2 | MATERIALS AND METHODS

2.1 | Chemicals and Reagents

All reagents and solvents were HPLC grade and were used as received. Mobile phase A consisted of an aqueous solution with 60 mM potassium perchlorate (Sigma Aldrich, St. Louis, MO). To each liter of this solution, 0.6 mL of 70% perchloric acid was added (Fisher Scientific, Waltham, MA). Mobile phase B was prepared by combining 60 mM potassium perchlorate with acetonitrile (Fisher Scientific) at a ratio of 45:55 (v:v), and 0.6 mL of a 70% perchloric acid solution was added to every liter. Samples and standards were prepared in diluent consisting of 20 mM ammonium formate (Sigma Aldrich) in water, adjusted with formic acid to pH 4.0.

Two oral solution samples purchased from the marketplace and designated as brand A and B, both containing phenylephrine and diphenhydramine at 1.25 and 0.50 mg/mL, respectively, were selected for the method development and validation. Sample solutions for

the impurity analysis were prepared by diluting original samples to 40% of their original concentrations using diluent. This resulted in diphenhydramine and phenylephrine nominal concentrations of 0.50 and 0.20 mg/mL, respectively. Spiked solutions were prepared in a similar manner, but impurities were added at 50, 100, and 150% of the proposed specification limits, which are reported in Table 1 for each impurity. Standards were received from United States Pharmacopeia (Rockville, MD) with the exception of phenylephrine related compound H (Toronto Research Chemicals, Ontario, Canada). For assay analysis, samples were diluted to 5% of the dosage strength, resulting in nominal concentrations of diphenhydramine and phenylephrine of 62.5 and 25 µg/mL, respectively. Spiked assay solutions were prepared by adding reference standards equal to 50, 100, and 150% of the nominal concentration.

2.2 | Liquid Chromatography

Unless otherwise stated, liquid chromatography was performed using an Agilent 1290 UHPLC (Agilent Technologies, Santa Clara, CA), and data was acquired and analyzed using Chemstation OpenLAB software (Agilent Technologies). This UHPLC was equipped with a 2.1×150 mm Kinetex column with $1.7 \mu\text{m}$ C8 core-shell particles (Phenomenex, Torrance, CA). For the evaluation of robustness and intermediate precision, chromatography was performed using a Waters Acquity UPLC (Waters, Milford, MA) and a 2.1×150 mm C8 Cortecs column with $1.6 \mu\text{m}$ superficially porous particles (Waters). For validation, all analyses were performed using a flow rate of 0.3 mL/min while columns were maintained at 15 °C (Table 2). The impurity method utilized 5 µL injections, while 4 µL injections were used for assay analysis. Detection occurred via aUV detector at 210 nm, and the gradient elution method is described in Table 3.

3 | RESULTS AND DISCUSSION

3.1 | Method Development

Two oral solution drug products (identified as A and B) were used during all phases of development to ensure that the final method was not specific to a single formulation. Development for the impurity method began using solutions and instrument parameters similar to those described in the USP tablet monograph for the same drug product combination [13]. The USP method had already demonstrated the ability to separate and quantify the impurities associated with the individual drug substances, and thus provided a starting point for development.

3.1.1 | Mobile Phase and Column Combination—Consistent with the tablet method, an aqueous 60 mM potassium perchlorate mobile phase was used as a weak solvent and acetonitrile was used as the strong solvent in combination with an alkyl amide column (L60 USP classification). Impurity spiked solutions (100% level) of each drug product were analyzed using a variety of instrumental conditions and mobile phase gradients, but the abundance of water soluble excipients in the oral solution samples prevented resolution of the early eluting phenylephrine impurities. This was the case for multiple brands and models of L60 columns, suggesting the amide stationary phase was insufficiently selective between the impurities and excipients. To address the lack of resolution, columns with different

stationary phases were investigated until resolution of the first four phenylephrine impurities was achieved using isocratic elution with the aqueous potassium perchlorate buffer. The best resolution was achieved using a Kinetex 2.1 × 150 mm column with 1.7 μm superficially porous C8 coated particles, thus, the remaining development was performed using this column.

3.1.2 | Selection of Remaining Chromatographic Parameters—To determine the optimum column temperature, injections of 100% impurity spiked solutions were performed while the column was held at a constant temperature between 15 and 40°C. Lower temperatures resulted in better resolution of phenylephrine related compound H, the earliest eluting impurity, from most of the water-soluble excipients. For this reason, 15°C was selected as the column temperature. Importantly, current USP specifications allow a ±10°C adjustment of the column temperature from the specified value [14]. This means that while the separation may perform better at subambient temperatures, the method could also be used without column cooling. At a temperature of 15°C, the maximum flow rate which resulted in pressures under the column limit (15,000 psi) was approximately 0.35 mL/min. Therefore, the flow rate for the method was lowered to 0.30 mL/min to ensure compatibility across UHPLCs from different vendors. Lastly, a UV detection wavelength of 210 nm was chosen to provide the required sensitivity for the diphenhydramine and phenylephrine impurities. While most excipients also absorbed at this wavelength, careful attention was given to ensure they did not co-elute with the impurities of interest. A complete summary of instrumental parameters is presented in Table 2.

3.1.3 | Mobile Phase Gradient—Chromatograms for standard solutions containing all impurities and sample solutions spiked with impurities at the concentrations equivalent to 100% of the limits as described in Table 1 are shown in Figure 1. These chromatograms were recorded using isocratic elution of mobile phase A, and they show resolution of phenylephrine and its related impurities H, G, F, and C for all solutions. Peaks before these compounds are attributed to the solvent front and excipients that elute prior to relevant impurities. From here, development proceeded by altering the mobile phase gradient until resolution of remaining impurities was achieved. Although separation of impurities in standard solution was easily obtained using a variety of simple gradients, the presence of excipients in spiked samples required a more complex gradient profile. Ultimately, a linear gradient program capable of separating all impurities from the respective drug substances and excipients in both drug samples was achieved. This program is reported in Table 3. This gradient can be viewed as two methods performed in series since diphenhydramine components do not significantly travel through the column until phenylephrine components have eluted. Viewed from this perspective, the phenylephrine separation begins with an isocratic hold (0–14 min), is followed by a linear gradient (14–31 min), and ends with a second isocratic hold (31–38 min). The diphenhydramine separation begins with an isocratic hold (38–44 min) and is stepped down to a second isocratic hold (54–66 min). The remaining steps are for the column wash (66–70 min) and regeneration (70–74 min). Chromatograms displaying the elution of the two remaining phenylephrine related compounds, D and E, are shown in Figure 2 for standard and spiked sample solutions. Elution of diphenhydramine and its related impurities is shown in Figure 3. Complete

chromatograms for the analysis of 100% spiked solutions using oral solution sample B (top) and oral solution sample A (bottom) are shown in Figure 4. Peaks not identified are attributed to excipients.

3.1.4 | Robustness Analysis—A robustness analysis was conducted to determine how well the separation was maintained under slightly different method conditions. This was critical in demonstrating that the use of a multi-step gradient could be performed reproducibly. First, the separation was performed using an UHPLC instrument produced by a different manufacturer with a different column brand than that used for development. A Waters Acquity UPLC utilizing a C8 Cortecs column with 1.6 μm superficially porous particles was used for this purpose (an Agilent 1290 UHPLC and Kinetex superficially porous C8 column were used to develop the method). Importantly, this instrument utilized a quaternary pumping system, while the original method was developed using a binary pump. Chromatograms for analysis of 100% spiked solutions were nearly identical to those displayed in Figures 1-4 which show resolution of all impurities and both drug compounds, indicating the separation was compatible with instruments and columns produced by multiple manufacturers. Resolution and retention times for all robustness conditions are available in the supporting information.

Next, instrument parameters were varied from their original values to ensure that the separation was not overly sensitive to changes in analysis parameters. Analysis was performed with modifications to the flow rate ($\pm 10\%$, 0.27 and 0.33 mL/min), temperature ($\pm 2^\circ\text{C}$, 13 and 17°C), injection volume ($\pm 2\ \mu\text{L}$, 3 and 7 μL), and the detection wavelength ($\pm 2\ \text{nm}$, 208 and 212 nm). Each condition was altered independently while the others remained identical to those used in the original method. Spiked solutions (100%) for each oral solution sample were analyzed, and the resolution of impurity and drug substance peaks were greater than 1.5 under all altered conditions, indicating baseline resolution (supporting information).

Lastly, each step of the gradient was altered by $\pm 2\%$ to test whether variation in mobile phase composition or differences in an instrument's dwell volume would lead to a failed separation. This was a critical part of the validation phase due to the multi-step gradient. Analysis of spiked solutions under each new gradient condition revealed that the separation remained successful (supporting information). Thus, no single method modification tested in the robustness analysis led to a failed separation.

3.2 | Validation of Impurity Method

Validity for impurity analysis was performed by demonstrating specificity, linearity, accuracy, precision, and limits of quantitation (LOQs) for each impurity. Experiments were performed using both drug products, but results are reported below for only the brand A (Table 4). Results for brand B samples showed similar outcomes and can be found in the supporting information.

3.2.1 | Specificity - Impurities—Injections of individual impurity standards were performed to confirm that retention times of different substances did not overlap. A 100%

spiked solution was then analyzed, and the resolution of impurity peaks was determined. Resolutions for all impurities were greater than 1.5, demonstrating specificity.

3.2.2 | Linearity - Impurities—Seven standard solutions were prepared ranging in concentration from 50 – 150% of the specification limit for each impurity. Solutions were analyzed and the correlation coefficients (r^2) of the regression curves were determined to be greater than 0.9900 for each analyte, demonstrating linearity.

3.2.3 | Accuracy - Impurities—Sample solutions spiked with impurities at 0 (nothing added), 50, 100, and 150% of the impurity specification level were prepared. This involved two preparations of unspiked solutions, six at the 50% level, and three each at the 100 and 150% levels. After analysis, recoveries in spiked solutions were calculated by comparing the experimentally determined and theoretical spiked impurity quantities. The average recovery percentage of all eleven impurities was within 80 – 120% for the 50% spiked solutions, between 90 – 110% for the 100% spiked solutions, and between 95 – 105% for the 150% spiked solutions. Representative chromatograms are available in the supporting information.

3.2.4 | Analysis Repeatability - Impurities—The relative standard deviation (RSD) of the recovery for the six 50% spiked solutions analyzed in the accuracy analysis was determined. Values for each impurity were less than 10%, indicating precision.

3.2.5 | Intermediate Precision - Impurities—For the intermediate precision analysis, two additional sample solutions (no impurities added) and six additional 50% spiked solutions were prepared and analyzed using a different UHPLC system (a Waters Acquity UPLC versus an Agilent 1290) with a different column (Cortecs versus Kinetex). The recovery of impurities in the new 50% spiked solutions was determined in the same manner as described in the impurity accuracy validation. The RSDs for the recovery of impurities for the new 50% spiked solutions were all less than 10% (supporting information). Additionally, when the recoveries for the original six spiked 50% solutions (analysis repeatability) and the new six spiked 50% solutions (intermediate precision) were combined, the overall RSDs for all impurities were under 20% (supporting information).

3.2.6 | Limits of Quantification - Impurities—Calibration curves acquired during the linearity analysis were used to determine LOQs using Equation (1), where s is the standard error and m is the slope calculated from regression.

$$LOQ = \frac{10 \times s}{m} \quad (1)$$

To compare results with impurity specification limits, values were converted to a percentage of the nominal drug concentration in sample solutions. These values, reported in Table 4, were approximately an order of magnitude less than impurity specification limits proposed for the USP monograph (Table 1). For example, diphenhydramine related compounds A and B had specification limits of 0.2%, and their LOQs were determined to be 0.02 and 0.03%, respectively.

3.3 | Validation of Assay Method

Using impurity method conditions, phenylephrine and diphenhydramine peaks displayed a large tailing factor (2.6 and 3.5, respectively), so two modifications to analysis parameters were made to reduce this effect for assay determination. First, the nominal concentration of sample solutions was lowered to 25 and 62.5 µg/mL for phenylephrine and diphenhydramine, respectively, which represented 5% of the drug concentration in the original product. Second, the injection volume was lowered from 5 to 4 µL. Together, these changes resulted in phenylephrine and diphenhydramine peaks with tailing factors of just 1.4 and 1.7, respectively.

Validation of the assay method was performed for both brands of drug products, but results are reported for only brand A (Table 5). The outcome for brand B samples was similar, and complete results are included as supporting information.

3.3.1 | Specificity - Assay—Specificity was demonstrated using critical pair resolution and peak purity analysis. Sample solutions were prepared and analyzed, and the critical pair resolutions of drug substances were determined. These values were 2.7 and 5.4 for phenylephrine and diphenhydramine, respectively, which were above the lower limit of 1.5. Next, a peak purity analysis was performed to determine if excipients were co-eluting with drug substances and altering absorbance measurements. UV spectra were collected from 200 – 410 nm at each time-point during phenylephrine and diphenhydramine peaks. Each spectrum within the peak was compared to that of the peak average, and a purity value was assigned based on the similarity of the spectral profile. The average purity value of both phenylephrine and diphenhydramine peaks were determined to be 999.9 out of a maximum 1000. The threshold for purity was 990.0, indicating that no substances were co-eluting with either drug substance. Additionally, spectra for phenylephrine and diphenhydramine standards closely resembled those obtained from sample solutions (spectra not shown).

3.3.2 | Linearity - Assay—Six standard solutions containing each drug substance at 50 – 150% of the nominal concentration were analyzed. Calibration curves for both substances had correlation coefficients exceeding 0.9990, demonstrating linearity.

3.3.3 | Accuracy - Assay—For accuracy validation, sample solutions were spiked with drug substance reference standards at 10, 20, and 30% of the nominal value, producing solutions that were approximately 110, 120, and 130% of the nominal concentration for each analyte. Solutions were prepared in triplicate, and the percent recovery of the spiked amount was determined. Average recoveries at each level were between 97.0 – 103.0%, indicating accuracy.

3.3.4 | Analysis Repeatability - Assay—Six preparations of sample solution were analyzed for their assay content. The RSDs of the drug substance content were 1.2 and 1.0% for phenylephrine and diphenhydramine, respectively. Both values were below the 2.0% acceptance value.

3.3.5 | Intermediate Precision - Assay—Similar to the impurity method validation, an alternative UHPLC system (Waters Acquity UPLC) and column (Cortecs) were used to

evaluate intermediate precision. Six new sample solutions were prepared and analyzed using the different instrumental setup, and the RSDs of the assay content for the new solutions were 1.2 and 1.0% for phenylephrine and diphenhydramine, respectively (supporting information). Additionally, the overall RSDs of pooled assay values for the analysis repeatability and intermediate precision analyses were determined to be 1.0 and 0.9% for respective analytes.

4 | CONCLUDING REMARKS

To our knowledge, this work is the first report of a single UHPLC method for the impurity determination of diphenhydramine and phenylephrine hydrochloride oral solutions. Six phenylephrine and five diphenhydramine impurities were separated from two drug substances and all excipients in two OTC formulations. A robustness study was conducted to show compliance across two UHPLCs from different manufacturers, and also that minor alterations to the analysis parameters did not compromise the separation. Methods were shown to be valid for impurity and assay determinations of diphenhydramine and phenylephrine hydrochloride oral solutions and may be considered for adoption as compendial procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Article Related Abbreviations:

FDA	United States Food and Drug Administration
ICH	International Council for Harmonisation
OTC	Over-the-counter
USP	United States Pharmacopeia

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FIGURE 1.

Partial chromatograms (0 – 12 min) for a 50% standard impurity solution (upper), 100% spiked solution of oral solution sample B (middle), and 100% spiked solution of oral solution sample A (lower). Asterisks denote the impurity peaks

FIGURE 2.

Partial chromatograms (28 - 37 min) of a 50% impurity standard solution (upper), 100% spiked solution of oral solution sample B (middle), and 100% spiked solution of oral solution sample A (lower). Asterisks denote the impurity peaks

FIGURE 3. Partial chromatograms (49 - 65 min) of a 50% impurity standard solution (upper), 100% spiked solution of oral solution sample B (middle), and 100% spiked solution of oral solution sample A (lower). Asterisks denote the impurity peaks

FIGURE 4.
Complete chromatograms of brand B (upper) and brand A (lower) 100% spiked solutions

TABLE 1

Proposed specification limits for impurities in diphenhydramine/phenylephrine oral solutions

Analyte	Abbreviation	Limit (%)
Diphenhydramine HCl	DPH	–
Diphenhydramine related compound A	RC A	0.2
Diphenhydramine related compound B	RC B	0.2
Diphenhydramine n-oxide	NOX	1.2
Benzophenone	BP	0.7
Benzhydrol	BH	0.5
Phenylephrine HCl	PHE	–
Phenylephrine related compound C	RC C	0.3
Phenylephrine related compound D	RC D	0.3
Phenylephrine related compound E	RC E	0.3
Phenylephrine related compound G	RC G	0.3
Phenylephrine related compound F	RC F	0.3
Phenylephrine related compound H	RC H	1.0

TABLE 2

Method parameters for impurity analysis

Flow rate	0.30 mL/min
Column Temperature	15 °C
Detection wavelength	210 nm
Injection Volume^a	5 µL
Column	2.1 × 150 mm with 1.7 µm Kinetex C8
Gradient	See Table 3

^aInjection volume was lowered to 4 µL for assay analysis

TABLE 3

Mobile phase gradient for analysis of sample solutions. Gradient changes are linear

Time (min)	A (%)	B (%)
0.00	100	0
14.00	100	0
14.01	80	20
17.00	75	25
31.00	67	33
38.00	67	33
38.01	55	45
44.00	55	45
54.00	40	60
66.00	40	60
66.01	0	100
70.00	0	100
70.01	100	0
74.00	100	0

TABLE 4

Impurity validation results for brand A oral solution

<i>Analyte</i>	<i>RT (min)</i>	Specificity <i>(Resolution)</i>	Linearity <i>(r²)</i>	Accuracy (Recovery)			Analysis Repeatability <i>(Recovery RSD)</i>		<i>LOQ (%)</i>
				<i>50%</i>	<i>100%</i>	<i>150%</i>			
RC H	5.50	4.1	0.9999	103.0	99.0	98.2	1.8		0.06
RC G	6.36	3.6	0.9997	100.3	102.2	104.2	1.4		0.03
RC F	7.48	5.3	0.9998	101.5	106.2	99.5	3.3		0.02
RC C	10.26	5.3	1.0000	104.4	106.0	104.8	5.2		0.01
RC D	32.27	7.9	0.9998	110.8	109.0	96.4	1.7		0.03
RC E	35.99	11.3	0.9997	100.3	103.7	104.2	1.0		0.03
RC B	50.14	44.3	0.9999	104.4	101.4	101.1	0.9		0.03
BH	52.58	7.8	0.9998	102.7	101.1	101.0	1.3		0.05
RC A	53.79	4.3	0.9999	98.0	98.4	99.2	1.1		0.01
NOX	59.02	9.2	0.9998	103.2	100.8	101.1	0.6		0.02
BP	63.25	9.3	0.9999	105.0	99.5	97.7	1.0		0.06
Limit	<i>N/A</i>	<i>1.5</i>	<i>0.9900</i>	<i>80 – 120%</i>	<i>90 – 110%</i>	<i>95 – 105%</i>	<i>10%</i>		<i>N/A</i>

TABLE 5

Assay validation results for brand A oral solution

<i>Analyte</i>	<i>RT (min)</i>	Specificity (<i>Resolution</i>)	Linearity (<i>r</i> ²)	Accuracy (Recovery)		Analysis Repeatability	
				110%	120%	130%	Assay RSD (%)
Phenylephrine	7.84	2.7	0.9999	100.3	99.5	100.9	1.2
Diphenhydramine	55.14	5.4	0.9999	100.3	99.3	100.8	1.0
Limit	<i>N/A</i>	<i>1.5</i>	<i>0.9990</i>	<i>97.0 - 103%</i>			<i>2.0%</i>