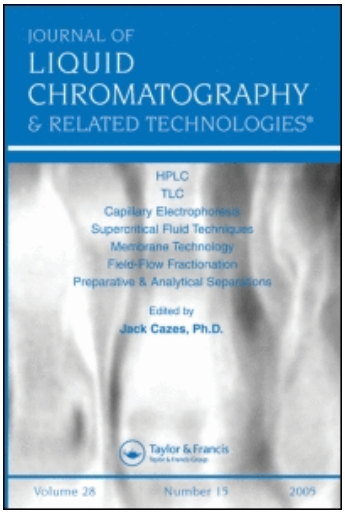

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Fast Determination of 4-Hydroxybenzoic Acid in Different Matrices using Monolithic Column Separation

Louis-Philippe Labranche[a]; Audrey Tousignant[a]; Daniel Abran[a]; Alain Carrier[a]
[a] Sandoz Canada Inc., Québec, Canada

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Fast Determination of 4-Hydroxybenzoic Acid in Different Matrices using Monolithic Column Separation

Louis-Philippe Labranche, Audrey Tousignant, Daniel Abran, and Alain Carrier

Sandoz Canada Inc., Québec, Canada

Abstract: Determination of impurity levels in injectable formulation is an important issue with regulatory agencies. Impurities associated with preservatives systems are questioned and the use of adequate tools and the accumulation of a large body of data are required to ensure the pharmaceutical quality of products. Liquid Chromatography combined with UV detection can be adapted to provide a powerful tool for impurity analyses in injectable solution formulations. Using an Onyx 100×4.6 mm monolithic column coupled with high flow rates (>5 mL/min) and UV detection at 254 nm, parabens synthetic pathway residue and degradation products were quantified in less than 1 minute. The analyses involved simple sample preparation and rapid run-time chromatography. Using these conditions, recoveries of 97.3 to 100.0% at three impurity levels (0.375, 0.750, and 1.125% of the active drug) were obtained for an injectable formulation containing indazole based active drugs. Injectables containing steroid and imidazole based active drugs were also tested. The standard and sample solutions were shown to be stable for at least 5 days when stored at 2–8°C. Tests demonstrated that the method is insensitive to small changes in experimental conditions. Data supporting the development and validation of this method are presented.

Keywords: 4-Hydroxybenzoic Acid, High-throughput chromatography, HPLC, Injectable solution, Methylparaben, Monolithic column, Propylparaben, Reversed phase chromatography, Validation

Correspondence: Dr. Alain Carrier, Sandoz Canada Inc., 145 Jules-Léger Boucherville, Québec, J4B 7K8 Canada. E-mail: alain.carrier@sandoz.com

INTRODUCTION

Parenterals are products intended for administration into the human body not by way of the alimentary canal. These products can either be solutions, suspensions, or emulsions in a suitable aqueous or non-aqueous vehicle.[1,2] In order to ensure the product safety and prevent inadvertent bacterial contamination of the formulation, preservatives are added to the finished dosage form. A group of preservatives commonly used in injectable solutions are 4-hydroxybenzoates (parabens). These chemicals have both bactericidal and fungicidal properties and are added to formulations in low concentrations requiring analysis by chromatography. Parabens are synthetized by esterification of a monomer of 4-hydroxybenzoic acid.[6] The general chemical structure of parabens is shown in Figure 1, where R corresponds to a methyl, ethyl, propyl, or butyl alkyl group. The methyl and propyl alkyl group are the most frequently used esters in injectable solutions due to their higher solubility in water when compared to the higher chain length parabens.[3,4] No direct evidence of allergic reactions to parabens have been demonstrated yet,[5] but the extensive presence of parabens in the pharmaceutical, cosmetic, and food industry ask for sensitive analytical methods to qualify and quantify the amount of parabens found in finished products.

Analytical procedures are being developed to analyze the parabens content in a variety of products. These methods should also monitor the parabens degradation products. Furthermore, they should be able to quantify 4-hydroxybenzoic acid originating from the synthesis or degradation pathways.

A variety of methods have been described to characterize 4-hydroxybenzoic acid. Liquid chromatography,[7–11,13–19] capillary electrochromatography (CE),[3,12] microemulsion electrokinetic chromatography,[6,12] and gas chromatography (GC)[7,9] have been used for qualitative and

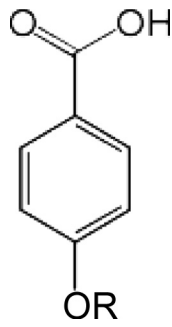

Figure 1. Representation of 4-Hydroxybenzoic acid.

quantitative determination of 4-hydroxybenzoic acid in different matrices. Of the several HPLC methods developed in the past, none are adequate for a rapid and routine determination and quantification of 4-hydroxybenzoic acid in different injectable matrices. The methods reported are highly specific to a given application and cannot be used for general determination in different formulations.

Monolithic columns have been introduced recently for use in HPLC.[20–22] Due to their highly porous silica pore structure, these columns are more permeable and result in lower operating pressure. Thus, they can efficiently separate complex mixtures at high flow rates and with high performance.

The objectives of the present study were the following: 1) develop a simple and rapid HPLC method for routine determination and quantification of 4-hydroxybenzoic acid in different injectable matrices using monolithic column, 2) provide comparative data to standard liquid chromatographic methods, 3) validate the method for injectable solution containing methylparaben and propylparaben as preservative and indazole molecule structure as active ingredients, and 4) test the versatility of the method on steroid or imidazole containing injectable solutions.

EXPERIMENTAL

Chemicals and Reagents

HPLC grade acetonitrile was purchased from Anachemia Canada Inc. (Montréal, QC, Canada). HPLC grade formic acid and 4-hydroxybenzoic acid were purchased from Sigma Aldrich (Montréal, QC, Canada). Distilled water available in the laboratory was filtered prior to use through a 0.2 μm filter.

LC Instrumentation, Columns, and Conditions

Analysis was carried out using an Agilent 1200 HPLC system (Agilent Technology, Canada) and a Rapid Resolution Agilent 1200 HPLC system (Agilent Technology, Canada). The HPLC system is composed of the following units: a solvent delivery module, an automatic sample injector, a column oven, and a UV detector programmed at 254 nm. The analysis was performed using a Phenomenex monolithic column Onyx^{TM} C_{18} (100 mm × 4.6 mm I.D), maintained at 40°C. Furthermore, Zorbax Eclipse XDB-C_{18} (75 mm × 4.6 mm I.D. (3.5 microns) and Zorbax Eclipse XDB-C_{18} (50 mm × 3.0 mm I.D. (1.8 microns) columns were used for development purposes. A 10 μL volume of the standard and sample solutions was injected into the HPLC system for analysis.

Sample Preparation

Standard solutions of 4-hydroxybenzoic acid (0.0003 mg/mL) and sample solutions of indazole active drug (0.04 mg/mL), steroid drug (0.04 mg/mL), and imidazole drug (0.04 mg/mL) combined with methylparaben and propylparaben preservatives were prepared by dissolving the appropriate amount of the pure reference standards and sample solutions in water.

RESULTS AND DISCUSSION

Method Development

An important consideration in the development phase of any analytical method is to appropriately define the application's usage and requirements. The parameters that needed to be controlled in the present situation included the chromatographic resolution, peak sensitivity, and analysis time. Our main objective was to enable rapid separation and quantification of the major degradation product of the parabens used as preservative in the injectable product, namely 4-hydroxybenzoic acid. This method could be used in the early development phases of a formulation or during the evaluation of the stability of a drug product.

HPLC methods normally use conventional reversed phase C_8 and C_{18} columns for the analysis of 4-hydroxybenzoic acid.[7,9–11,13,15–19] For comparative purposes, our initial development work was done using a conventional Zorbax C_{18} 75 mm × 4.6 mm column with 3.5 μm particle size. The mobile phase used a mixture of 0.1% formic acid in water and acetonitrile. From the various mobile phases tested, the best conditions were 95% of 0.1% formic acid in water and 5% of acetonitrile. Tests were done to increase the organic content up to 5% to reduce the retention time of p-hydroxybenzoic acid but the results were not conclusive. Under optimized conditions, the total runtime combining the separation (3 min), the washing (2 min), and the reconditioning (5 min) steps was approximately 10 minutes. The reconditioning step comprised 10 column volumes. In order to reduce the total run time, the flow rate was increased but unfortunately, the resulting operating pressure was too high. The final optimized conditions using a flow rate of 2.0 mL/min and a composition of 95% of 0.1% of formic acid in water and 5% of acetonitrile gave a total run time of 10 minutes.

The next step was to take these optimized conditions and combine them with the high pressure advantage of the Agilent 1200 rapid resolution system. A typical rapid resolution column, the Zorbax Eclipse XDB-C_{18} (50 mm × 3.0 mm I.D., 1.8 microns particle size) was used. Again, optimization indicated that the best conditions found remained 95% of 0.1%

Table 1. Columns and final conditions used in method development

Chromatographic conditions: Mobile phase: 95% 0.1% Formic Acid/5% ACN	Analysis time (min)	Washing time (min)	Conditioning time (min)	Total run time (min)
Columns tried				
Zorbax Eclipse-XDB C_{18}, 75×4.6 mm (3.5 microns) Flow rate: 2.0 mL/min	3	2	5	10
Zorbax Eclipse-XDB C_{18}, 50×3.0 mm (1.8 microns) Flow rate: 1.0 mL/min	2	2	4	8
Onyx C_{18}, 100×4.6 mm Flow rate: 5.8 mL/min	1	1	1	3

formic acid in water and 5% of acetonitrile. No improvement was observed by varying the composition of the mobile phase, and the peak shape deteriorated with increased flow rate, even though improvement of the total run time was observed. Using the optimized conditions (95% of 0.1% of formic acid in water and 5% of acetonitrile) at a flow rate of 1.0 mL/min, the total runtime combining the separation (2 min), the washing (2 min), and the reconditioning (4 min) steps was around 8 minutes.

In order to minimize analysis time, we took advantage of the high permeability of the Phenomenex Onyx^{TM} C_{18} (100 mm $\times$ 4.6 mm I.D) monolithic column. The possibility of increasing the flow rate was the key factor in choosing the monolithic column. Flow rates ranging from 3 to 5.8 mL/min were tested with the previously optimized chromatographic conditions. Our results demonstrated that peak profiles and separation were still adequate even at a flow rate of 5.8 mL/min. Under these conditions, the total runtime was approximately 3 minutes. It was noted that the reconditioning step correspond to 4 column volumes but considering the delay between each injection, the column conditioning is still adequate. The method met our initial requirements of rapidly getting information regarding the degradation of parabens. A summary of the tested conditions is presented in Table 1 and the final chromatographic conditions can be found in Table 2.

Validation

The method developed for the determination of 4-hydroxybenzoic acid in injectable solution (containing indazole, methylparaben, and

Table 2. Method for the determination of 4-Hydroxybenzic acid in injectable solutions

Parameters	Proposed conditions		
Mobile phase (A)	0.1% Formic acid		
Mobile phase (B)	Acetonitrile		
Diluting solution	Water		
Column	Onyx®, 100 × 4.6 mm i.d. (Phenomenex)		
Column temperature	40°C		
Flow	5.8 mL/min		
Gradient	(min)	A	B
	0	95	5
	1.0	95	5
	1.1	50	50
	2.0	50	50
	2.1	95	5
	3.0	95	5
Detector	254 nm		
Injection volume	10 µL		
Test concentration	~0.0003 mg/mL as 4-Hydroxybenzoic acid ~0.04 mg/mL as Active drug		
Retention time 4-Hydroxybenzoic acid	0.9 minute		

propylparaben) has been validated. The validation parameters are presented in the following sections. All data were generated using an HPLC system from Agilent (1200 Series).

Specificity

The specificity of the method was tested by injecting a diluting solution blank and a solution containing all ingredients except methylparaben and propylparaben considering that 4-hydroxybenzoic acid is a degradation product of these molecules. No peaks were observed at the retention time of 4-hydroxybenzoic acid. A standard solution of 4-hydroxybenzoic acid was injected and a peak was observed at 0.9 minute. A sample preparation of the injectable solution containing indazole active drug and methylparaben and propylparaben as preservative was then injected. The 4-hydroxybenzoic acid peak was observed at 0.9 minute. Figures 2 and 3 are representative chromatograms of a standard solution of 4-hydroxybenzoic acid (A) and of a representative sample preparation solution of indazole active drug and methylparaben/propylparaben as preservatives (B).

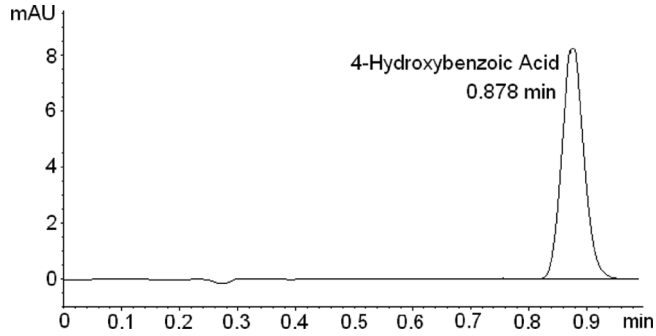

Figure 2. Standard solution containing 4-Hydroxybenzoic acid equivalent to approximately 0.002 mg/mL.

Linearity

Eight (8) standard solutions containing 4-hydroxybenzoic acid at concentrations of 0.00003 mg/mL to 0.0006 mg/mL representing 10% – 200% of the standard solution were prepared and injected. Details of the calibration curve results are presented in Table 3.

Range and Precision

The method was found to be precise, accurate, and linear at concentrations ranging from 0.00015 mg/mL to 0.00044 mg/mL of 4-hydroxybenzoic acid. This represents approximately 50% to 150% of the set limit for 4-hydroxybenzoic acid (0.0003 mg/mL). The precision of the system was evaluated from six (6) replicate injections of the 4-hydroxybenzoic acid standard solution at 0.0003 mg/mL. Typical results are as follows: Tailing factor ($T \leq 2$) = 1.3 with a RSD of 0.42%.

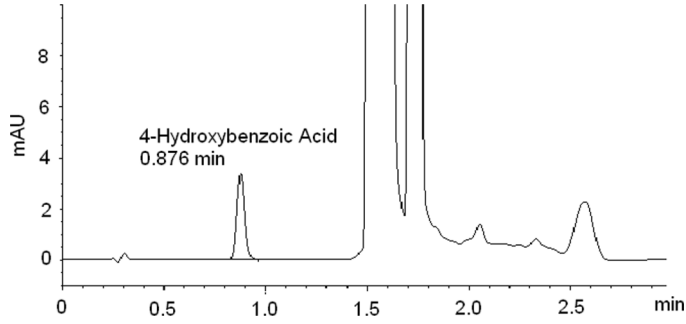

Figure 3. Sample preparation of injectable solution (indazole active drug and methylparaben/propylparaben as preservatives) containing 4-Hydroxybenzoic acid.

Table 3. Coefficient of determination, slope and intercept of 4-Hydroxybenzoic acid

Compound	Coefficient of determination (R^2)	Slope	Intercept
4-Hydroxybenzoic acid	0.9997	20305	0.0452

Accuracy

The synthetic finished product was spiked at 50%, 100%, and 150% of the set limit for 4-hydroxybenzoic acid representing approximately 0.00015 mg/mL (0.375%), 0.0003 mg/mL (0.750%), and 0.00045 mg/mL (1.125%). Results are reported in Table 4. The overall average recovery for 4-hydroxybenzoic in injectable formulation (containing indazole active drug and methylparaben/propylparaben excipients) is 99.1% with a recovery range of 97.3% to 100.0%.

Table 4. Synthetic injectable solution (indazole active drug and methylparaben/propylparaben as preservatives) containing 4-Hydroxybenzoic acid at 50%, 100%, and 150% of the set limit

Accuracy	Theoretical conc. (mg/mL)	Experimental conc. (mg/mL)	Recovery (%)	Average recovery (%)	RSD (%)
50%	0.00015	0.00015	100.0	100.0	0
	0.00015	0.00015	100.0		
	0.00015	0.00015	100.0		
	0.00015	0.00015	100.0		
	0.00015	0.00015	100.0		
	0.00015	0.00015	100.0		
100%	0.00030	0.00029	96.7	97.3	1.4
	0.00030	0.00029	96.7		
	0.00030	0.00030	100.0		
	0.00030	0.00029	96.7		
	0.00030	0.00030	96.7		
	0.00030	0.00029	96.7		
150%	0.00044	0.00044	100.0	100.0	0
	0.00044	0.00044	100.0		
	0.00044	0.00044	100.0		
	0.00044	0.00044	100.0		
	0.00044	0.00044	100.0		
	0.00044	0.00044	100.0		

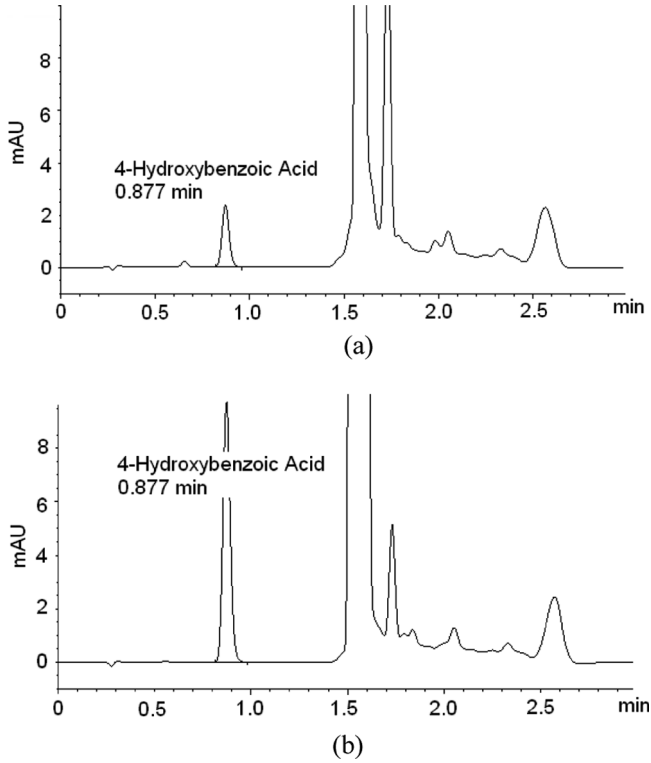

Figure 4. (a) Sample preparation of injectable solution (steroid active drug and methylparaben/propylparaben as preservatives). (b) Sample preparation of injectable solution (imidazole active drug and methylparaben/propylparaben as preservatives).

Stability of Solutions

A standard solution of 4-hydroxybenzoic acid was re-analysed after 1, 2, and 5 days and compared to a freshly prepared standard solution. The results showed no significant decrease of 4-hydroxybenzoic acid after 5 days (99.5% recovery) when stored at room temperature and after 5 days (99.9% recovery) when stored at 2–8°C. In the same manner, a sample preparation of injectable solution (containing indazole active drug and methylparaben/propylparaben excipients) was found to be stable at least 5 days when stored at 2–8°C (101.6% recovery).

Method Robustness

Robustness was measured by varying the column temperature by ±2°C and the percentage organic in the mobile phase by ±2%. The retention

time and the peak shape were not affected by any of the changes tested. Therefore, the proposed method is found to be robust over variations of $\pm 2^{\circ}C$ of the column temperature and variations of $\pm 2\%$ organic in the mobile phase composition.

Versatility of the Method

Additional studies were carried out to assess whether they can be applied to other injectable solutions containing methylparaben and propylparaben combined with steroid or imidazole molecules as active drugs. Each sample was tested and chromatograms are presented in Figures 4a and 4b.

CONCLUSION

A simple and rapid reversed phase HPLC method using a monolithic column was developed for routine analysis of 4-hydroxybenzoic acid in injectable solution. Different types of injectable formulation containing active drugs, such as indazole, steroid and imidazole and containing parabens as preservatives, can be analyzed with this method.

The proposed method was validated and exhibits no significant peak interferes with the 4-hydroxybenzoic acid peak. The recovery of 4-hydroxybenzoic acid is 97.3% to 100.0% from synthetic solutions at three different levels of the set limit of 4-hydroxybenzoic acid (50%, 100%, and 150%). 4-Hydroxybenzoic acid shows a linear response with a coefficient of determination (R^2) of 0.9997 in the range of 10% to 200% of the set limit concentration. The standard and sample solutions are stable for 5 days when stored at 2–8°C. The method is relatively insensitive to small changes in experimental conditions. In conclusion, this simple and rapid HPLC method used for the analysis 4-Hydroxybenzoic acid in injectable solution (containing indazole active drug and methylparaben/propylparaben as preservatives) has been validated and is acceptable for its intended use.

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