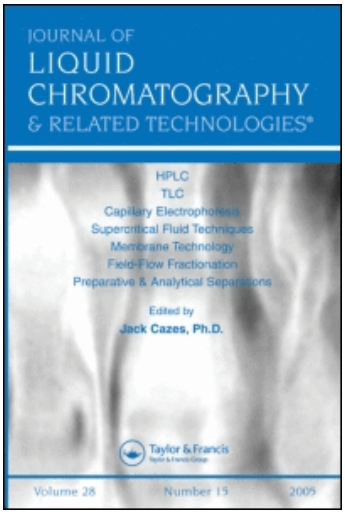

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Method Development and Validation of Preservatives (Phenylformic Acid, 2,4-Hexadienoic Acid, Methyl 4-Hydroxybenzoate, and Propyl 4-Hydroxybenzoate) by HPLC

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To cite this Article Shabir, Ghulam A. , Lough, W. John , Arain, Shafique A. and Shar, Ghulam Qadir(2006) 'Method Development and Validation of Preservatives (Phenylformic Acid, 2,4-Hexadienoic Acid, Methyl 4-Hydroxybenzoate, and Propyl 4-Hydroxybenzoate) by HPLC', Journal of Liquid Chromatography & Related Technologies, 29: 9, 1223 — 1233

To link to this Article: DOI: 10.1080/10826070600598399

URL: http://dx.doi.org/10.1080/10826070600598399

Journal of Liquid Chromatography & Related Technologies®, 29: 1223–1233, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600598399

Method Development and Validation of Preservatives (Phenylformic Acid, 2,4-Hexadienoic Acid, Methyl 4-Hydroxybenzoate, and Propyl 4-Hydroxybenzoate) by HPLC

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Abstract: A high performance liquid chromatography (HPLC) method for the assay of phenylformic acid, 2,4-hexadienoic acid, methyl 4-hydroxybenzoate, and propyl 4-hydroxybenzoate preservatives was developed and validated. The use of a reversed-phase C_8 column using an isocratic elution system enabled four compounds to be separated simultaneously in one analytical run. The method was validated to demonstrate its selectivity, linearity, precision, accuracy, specificity, and robustness. The calibration curves showed good linearity for all four analytes over the concentration range 0.026 to 4000 μg/mL. The correlation coefficients were in all cases greater than 0.999. The mean percent relative standard deviation values for precision

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studies were less than 2% in each case. This method represents a useful protocol for routine testing of preservatives.

Keywords: HPLC, Method validation, Preservatives, Phenylformic acid, 2,4-Hexadienoic acid, Methyl 4-hydroxybenzoate, Propyl 4-hydroxybenzoate

INTRODUCTION

Pharmaceutical preservatives contribute to the safe and stable supply of drugs, food products, and raw materials. Three primary classes of these compounds are known: antimicrobials, antioxidants, and chelating agents.[1] Preservatives, some of them naturally occurring, are used in a wide range of applications to maintain overall product quality. Some preservatives act as antimicrobial agents, some act as antioxidants, and some can perform both functions. The ability of a chemical to act as a preservative depends very much on the environment, so that factors such as type of the product, water content, pH, and storage conditions need to be considered when selecting a preservative. Antimicrobials and antioxidants are added to pharmaceutical products to prolong shelf life and maintain sterility.

The most commonly used preservatives in drug formulations are phenylformic acid (also called benzoic acid, BA, Figure 1a), 2,4-hexadienoic acid (sorbic acid, SA, Figure 1b), methyl 4-hydroxybenzoate (methyl paraben, MP, Figure 1c), and propyl 4-hydroxybenzoate (propyl paraben, PP, Figure 1d). Excess amount of these additives can be harmful to human

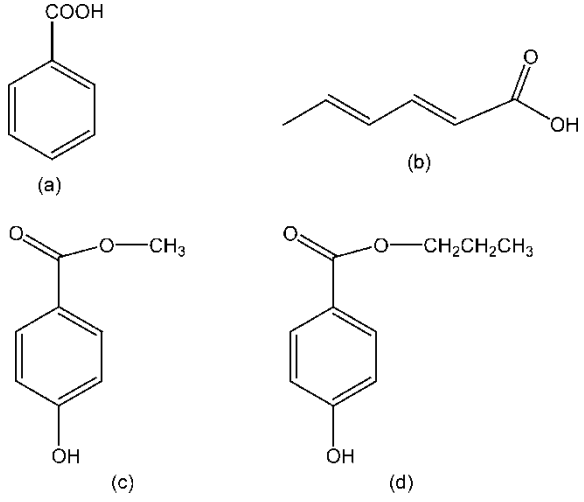

Figure 1. Structures of the analytes, in order of elution: (a) phenylformic acid; (b) 2,4-hexadienoic acid; (c) methyl 4-hydroxybenzoate; (d) propyl 4-hydroxybenzoate.

health. Therefore, minimum permissible concentrations of BA, SA, MP, and PP are controlled by regulation, and the quantitative analysis of these preservatives is important in routine analysis of pharmaceutical products.

Numerous cases of adverse reactions to parabens and benzoates in sensitive individuals have been reported in the literature.[2,3] Parabens and benzoates are often considered collectively in terms of their sensitivity reactions due to structural similarities. Parabens, alkyl esters of *p*-hydroxybenzoic acid, are a class of antimicrobial agents, particularly useful against molds and yeast. Parabens are effective over a wide pH range but more active in acidic conditions. Maximum chemical stability is at pH 4 to 5. Various combinations of MP and PP are used to give a total concentration of about 0.1% w/v. Phenylformic acid is a mono-functional, aromatic acid, which is widely used as a preservative in the cosmetic, drug, and food industries. Phenylformic acid is employed in a wide range of preservative applications because of its combination of bactericidal and bacteriostatic action with non-toxicity and tastelessness. It is the most effective preservative against yeast and mould. It is used in concentrations of 0.05–0.1%, and is active at a pH of 4.5 or lower; at values above pH 5 phenylformic acid is almost inactive. 2,4-Hexadienoic acid is widely used as a preservative in pharmaceutical liquids and semi-solids formulations 0.05–0.2%. In view of its limited stability and activity against bacteria, 2,4-Hexadienoic acid is frequently used in combination with other antimicrobials or with glycols where synergistic effects appear to take place.

The analytical determination of these preservatives is not only important for quality assurance purposes but also for consumer interest and protection. The most common analytical method for the determination of BA and SA, or the parabens, has been reversed-phase HPLC,[4–8] although other analytical methods such as TLC,[9] capillary electrophoresis,[10] and gas chromatography[11] have also been reported.

Most of the reported methods are for the separation of BA and SA, or amongst the parabens. However, chromatographic assay reports on the simultaneous determination of BA, SA, and parabens are scarce.

Such a method is important, as there seems to be an increasing trend in using combinations of preservatives, not only in the food industry but also in pharmaceutical formulations and cosmetic products.[6,12] Therefore, the objective of this study was to develop and validate a simple, accurate, and rugged reversed-phase HPLC method for the assay of simultaneous determination of a mixture of phenylformic acid, 2,4-hexadienoic acid, methyl 4-hydroxybenzoate, and propyl 4-hydroxybenzoate.

Validating analytical methods is a crucial component of successful product development, testing, and quality. All product types require some level of evaluation and testing either at the raw material, intermediates, or final product level. Critical decisions may be made based on these results, making it imperative that pharmaceutical and other companies ensure their accuracy and reproducibility remain compliant with regulatory guidelines in

the current climate of increased enforcement. The step-by-step written and approved protocol for test method validation should be followed.[13]

EXPERIMENTAL

Chemicals and Reagents

Phenylformic acid (99.5%), 2,4-hexadienoic acid (99%), methyl 4-hydroxybenzoate (99%), propyl 4-hydroxybenzoate (99%), and ammonium acetate (98%), were obtained from Sigma (St. Louis, MO, USA). Acetic acid (99.8%), and HPLC grade methanol were obtained from Merck (Darmstadt, Germany). Deionized distilled water was used throughout the experimental study.

HPLC Instrumentation

The analytical separations were carried out on a Perkin Elmer (Norwalk, CT) HPLC system, equipped with a model LC 235C diode array detector (DAD), series 200 LC pump, series 200 autosampler, and series 200 peltier LC column oven, using a Hypersil C_8 column (250 × 4.6 mm, 5 μm) at 30°C. The mobile phase was acetate buffer (20 mM, pH adjusted to 4.2 using acetic acid)/methanol (65:35, v/v). The mobile phase was filtered through a 0.45 μm membrane filter and degassed before use. The flow rate was set at 1.0 mL/min. UV detection was performed at 254 nm and the volume of sample injected was 20 μL.

Preparation of the Standard and Sample Solutions

Stock solutions of each of the standards (phenylformic acid, 2,4-hexadienoic acid, methyl 4-hydroxybenzoate, and propyl 4-hydroxybenzoate) were prepared in water at a concentration of 1 mg/mL. Further dilutions were made in the mobile phase. Linearity experiments were performed by preparing each preservatives (phenylformic acid, 2,4-hexadienoic acid, methyl 4-hydroxybenzoate, and propyl 4-hydroxybenzoate) standard solution in the range of 2-4000, 0.033-162, 0.026-168, and 0.197-491 μg/mL, respectively.

RESULTS AND DISCUSSION

Chromatography

Some chromatographic parameters such as column type, mobile phase, and conditioning time, were investigated to obtain a good separation of the four

analytes within acceptable time span in one HPLC analytical run. Different from other related methods,[4–8] in which C_{18} stationary phases are employed, a C_8 column was selected here to obtain good separation in a relatively short time. With this column, the percentage of the methanol (between 10 and 20%) and the pH values (in the 3.5–4.5 range) of the mobile phase were studied. Best chromatographic performance was obtained with a mobile phase at pH 4.2 containing methanol (35%). At least 30 min was necessary to reach equilibrium between the mobile phase and the stationary phase. If samples were injected earlier, retention times varied considerably, thus impairing correct peak identification.

The wavelength chosen for UV detection must provide acceptable absorbance for the various analytes in the sample, combined with acceptable light transmittance by the chosen mobile phase. For some samples, it may also be important to select a wavelength at which sample interferences have minimal absorption. Figure 2 shows the UV absorption spectra of four preservative compounds (BA, SA, MP, and PP).

It could be anticipated that a small peak for BA would be obtained if the detector wavelength was fixed at 254 nm, while on the other hand, small peaks for SA, MP, and PP would be obtained if the detector was set at 230 nm. Thus, in order to obtain maximum sensitivity, detection at the respective maximum wavelength of the preservatives can be done (e.g., 230 nm for BA, 254 nm for the others). However, here SA, MP, and PP were of most interest, so 254 nm was chosen as the optimal wavelength for maximum detection sensitivity. A system suitability test was developed for the routine application of the assay method.

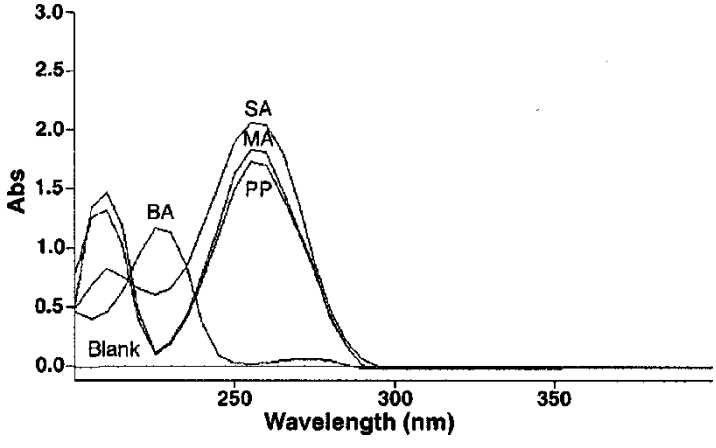

Figure 2. DAD UV spectra for four preservatives (phenylformic acid (benzoic acid, BA); 2,4-hexadienoic acid (sorbic acid, SA); methyl 4-hydroxybenzoate (methyl paraben, MP); propyl 4-hydroxybenzoate (propyl paraben, PP).

Under the stated experimental conditions, baseline resolution of the four components was achieved. The resolution between the peaks of 2,4-hexadienoic acid and phenylformic acid was 2.59, and methyl 4-hydroxybenzoate and 2,4-hexadienoic acid was 3.25. The retention times for BA, SA, MP, and PP were about 4.1, 5.0, 6.2, and 24.0 min (Figure 3); tailing factor (T) 1.04, 1.08, 1.09, and 1.05, respectively. The theoretical plate number (N) $\geq$3,200; retention time variation RSD $<$0.32%, and the RSD of peak areas were 0.72% for six injections in each case.

For the determination of method robustness within a laboratory during method development, a number of chromatographic parameters were evaluated such as flow rate, column temperature, different room temperature, mobile phase composition, pH, flow rate injection volume, columns from different batches, different equipment, and the quantitative influence of the variables were determined. For each parameter studied, three injections of both standard and sample solutions were injected. In all cases the influence of the parameters were found to be within a previously specified tolerance range. This showed that the method for determination of BA, SA, MP, and PP was reproducible and robust.

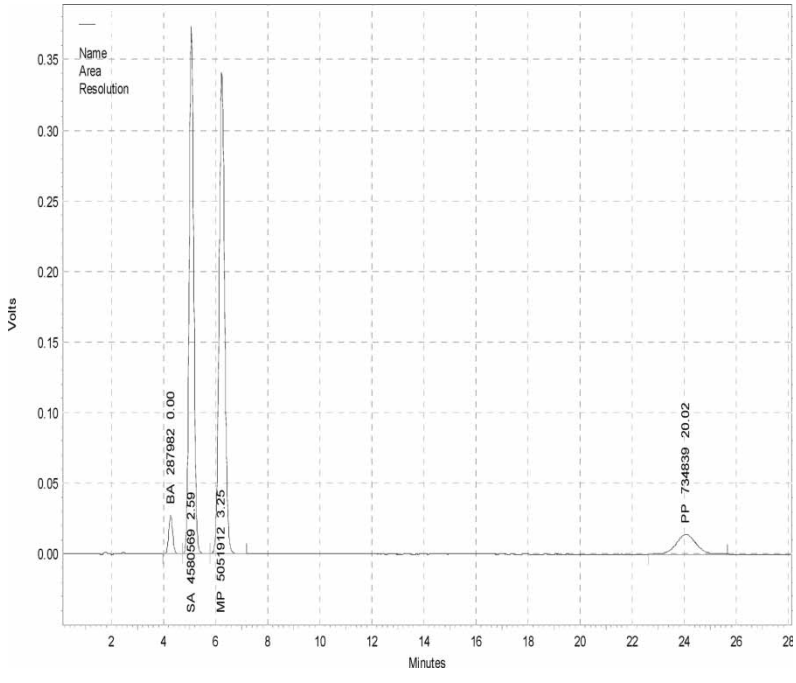

Figure 3. HPLC chromatogram of the preservatives (phenylformic acid (benzoic acid, BA); 2,4-hexadienoic acid (sorbic acid, SA); methyl 4-hydroxybenzoate (methyl paraben, MP); propyl 4-hydroxybenzoate (propyl paraben, PP).

Validation of the Chromatographic Method

Linearity

Linearity was studied using five solutions in the concentration range 0.026 to 4000 μg/mL ($n = 3$). The regression equation was found by plotting the peak area (y) versus the preservatives concentration (x) expressed in μg/mL. The correlation coefficients ($r^2 = 0.999$) obtained in each case for the regression line demonstrates that there is a strong linear relationship between peak area and concentration of preservatives (Table 1).

Accuracy

The accuracy of an analytical method is determined by how close the test results obtained by that method come to the true value. It can be determined by application of the analytical procedure to an analyte of known purity (for the drug substance) or by recovery studies, where a known amount of standard is spiked in the placebo (for the drug product). In the present study, a number of different solutions were prepared with a known added amount of each preservative and injected in triplicate. Percent recoveries of response factor (area and concentration) were calculated as can be seen in Table 2, and it is evident that the method is accurate within the desired range.

Precision

The precision of the chromatographic method, reported as %RSD, was estimated by measuring repeatability (intra-day assay precision) on ten replicate injections at 100% test concentration. Intermediate precision (inter-day variation) was demonstrated by two analysts using two HPLC systems, and evaluating the relative peak area percent data across the two HPLC systems at three concentration levels (50, 100, and 150%). The

Table 1. Linearity assessment of the HPLC method for the assay of preservatives, peak area vs. concentration ($n = 5$)

Preservatives	Concentration (μg/mL)	Equation for regression line	Correlation coefficient
Phenylformic acid	2–4000	$Y = 5.6498x + 125.26$	0.9990
2,4-hexadienoic acid	0.033–162	$Y = 133.59x + 109.54$	0.9999
Methyl 4-hydroxybenzoate	0.026–168	$Y = 165.52x + 118.13$	0.9993
Propyl 4-hydroxybenzoate	0.197–491	$Y = 145.59x + 695.24$	0.9993

Table 2. Recovery studies

	Applied concentration (% of target)		
Preservatives	50	100	150
Phenylformic acid	98.4 (±0.8)[a]	96.1 (±1.6)	94.3 (±2.6)
2,4-Hexadienoic acid	97.1 (±1.4)	96.6 (±1.9)	95.0 (±2.3)
Methyl 4-hydroxybenzoate	97.8 (±0.7)	97.1 (±1.7)	96.3 (±2.1)
Propyl 4-hydroxybenzoate	99.2 (±0.6)	98.6 (±0.8)	97.3 (±2.2)

[a]The coefficient of variation.

%RSD values presented in Table 3 were less than 2% in all cases, and illustrated the good precision of the analytical method.

Limit of Detection and Quantification

The limit of detection (LOD) and quantitation (LOQ) tests for the procedure were performed on samples containing very low concentrations of analyte. LOD is defined as the lowest concentration of analyte that can be detected above baseline noise. Typically, this is three times the noise level. LOQ is defined at the lowest concentration of analyte that can be reproducibly quantitated above the baseline noise with a signal to noise of 10. In this study, the LOD for BA, SA, MP, and PP was 0.5, 0.1, 0.3, 0.1 μg/mL and the LOQ was 2.0, 0.3, 0.8, and 0.3 μg/mL, respectively (Table 3).

Specificity

Forced degradation studies were performed to evaluate the specificity of each preservative under four stress conditions (heat, UV light, acid, base). Solutions of each preservative were exposed to 50°C for 1 h, UV light using a Mineralight UVGL-58 light for 24 h, acid (1M HCl) for

Table 3. Method validation results

	Preservatives			
Parameter	BA	SA	MP	PP
LOD (μg/mL)	0.5	0.1	0.3	0.1
LOQ (μg/mL)	2.0	0.3	0.8	0.3
Repeatability (Peak area %RSD, $n = 10$)	0.98	0.88	0.79	1.09
Intermediate precision ($n = 3$) instruments %RSD	1.59	1.96	1.40	1.72
Analysts %RSD	1.36	1.65	1.22	1.56

Table 4. Assay (%) of preservatives under stress conditions

Stress conditions	Sample treatment	RT (min) (BA, SA, MP, PP)	Assay (%) (BA, SA, MP, PP)
Reference	Fresh solution	4.14, 5.03, 6.21, 24.08	96.20, 96.51, 97.32, 98.41
Acid	1 M HCl for 24 h	4.13, 5.02, 6.20, 24.07	96.23, 96.52, 97.35, 98.44
Base	1 M NaOH for 4 h	4.14, 5.03, 6.21, 24.08	96.12, 96.46, 97.23, 98.33
Heat	50°C for 1 h	4.14, 5.03, 6.21, 24.08	96.16, 96.49, 97.30, 98.38
Light	UV light for 24 h	4.13, 5.02, 6.20, 24.08	96.10, 96.21, 97.17, 98.27

24 h, and base (1M NaOH) for 4 h. A summary data of the stress results is shown in Table 4, which showed no changes in retention times of each preservative and no degradation peaks were detected. This was further confirmed by peak purity analysis on a DAD UV detector and, therefore, confirms the specificity of the method.

Measurement of Robustness

During robustness testing, a method must prove to be able to remain unaffected by small, but deliberate variations in method parameters, thus showing its own reliability during normal usage. It is advisable to simultaneously study the possible variations of method parameters in an interval chosen symmetrically around the optimised conditions. This interval represents the variations expected during method transfer and routine use in quality control testing. In this case, the six selected parameters were the

Table 5. Experimental domain of the factors during robustness testing

Factor	Experimental domain	Optimised conditions
Buffer conc. (mM)	19.5–20.5	20
Flow rate (mL/min)	0.8–1.2	1.0
Injection volume (μL)	18–22	20
pH	3.7–4.7	4.2
Percent organic solvent	33–37	35
T (°C)	28–32	30

same considered in the optimisation step. Their experimental domain is reported in Table 5. This showed that the method for determination of BA, SA, MP, and PP was reproducible and robust.

A system suitability test was performed to determine the accuracy and precision of the system by injecting six replicate injections of preservative standard solutions. The RSD of the peak areas responses was measured. The %RSD of peak areas averaged was $\leq 0.72\%$ ($n = 6$) for each preservative.

CONCLUSIONS

This method for assaying phenylformic acid, 2,4-hexadienoic acid, methyl 4-hydroxybenzoate, and propyl 4-hydroxybenzoate is capable of high throughput. The validation study showed good linearity, sensitivity, accuracy, and precision. The suggested technique can be used in quality control for release of incoming raw material of phenylformic acid, 2,4-hexadienoic acid, methyl 4-hydroxybenzoate, and propyl 4-hydroxybenzoate preservatives used in pharmaceutical products.

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Received September 12, 2005
Accepted October 21, 2005
Manuscript 6735