Development and validation of liquid chromatographic methods for the estimation of the acceptance values of some hazardous preservatives in pharmaceutical formulations. A comparative study

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ABSTRACT

Gradient and isocratic liquid chromatography were applied for the simultaneous determination of three preservatives, namely; benzyl alcohol (BA), methyl paraben (MP) and propyl paraben (PP). Separation for both elution modes was carried out using a C18 column kept at 60°C throughout the analysis, accompanied with a flow rate of 2 mL/min and UV detection at 254 nm. Gradient liquid chromatography used a time programme with two solvents (0.1% formic acid and acetonitrile), while isocratic elution was performed with 0.1% formic acid: 2-propanol in a ratio of 70:30, v/v. Full validation study was conducted for both separation modes, where both of them were linear over the concentration ranges of 100.0–2000.0, 2.5–100.0, and 2.0–200.0 μg/mL for BA, MP and PP respectively. Using gradient elution, the detection limits for BA, MP, and PP were found to be 50.0, 1.8, and 1.5 μg/mL respectively. The corresponding values in isocratic elution were 60.0, 1.5, and 1.3 μg/mL. On the other hand, the limit of quantification for BA, MP, and PP using gradient mode are 70.0, 2.0, and 1.8 μg/mL respectively. While isocratic elution resulted in corresponding values of: 80.0, 2.3, and 1.7 μg/mL. The accuracy of the proposed method was illustrated from the high percentage recoveries values which were 99.67%, 100.42%, and 99.85% for BA, MP, and PP respectively using gradient mode, with corresponding values of 99.79%, 100.39%, and 99.87% for the isocratic elution mode. In addition, gradient elution was further applied to detect and assay the selected preservatives in different pharmaceutical formulations demonstrating that their quantities are within the stated ranges, and eventually, a comparative overview was introduced.

1. Introduction

The United States Pharmacopoeia [1] classifies the analytical procedure required to assay both major bulk drugs and inactive ingredients including preservatives, as category I under the elements necessary to carry out validation. Contribution of preservatives to the safety and stability of pharmaceutical products, food stuff, and health care products, cannot be ignored. The main role of preservatives is to act as an antimicrobial or/and antioxidant to maintain the quality of the final product [2], elongating its shelf life, and accomplishing sterility. Such valuable role ensures consumer satisfaction and protection [2].

Many factors affect the capability of a certain chemical to act as a preservative, such factors include: medium pH, water percentage, stated storage conditions, and the type of the manufactured product; since the used preservative should be compatible with other components in the formula [2].

This study is concerned with two examples of p-hydroxy benzoic acid esters (parabens), namely; methyl p-hydroxy benzoate (methyl paraben; MP), and propyl p-hydroxy benzoate (propyl paraben; PP); in addition to benzyl alcohol (BA) (Figure 1), since they are used extensively as antimicrobial products in pharmaceuticals in the Egyptian market. This fact gives us the motivation to estimate their concentration in various parenteral and liquid dosage forms.

It is reported in the literature that long term consumption of parabens affect the reproduction adversely [3], while levels of BA in injectable formulations exceeding the stated ones causes toxicity [4]. Upon using these preservatives in parenteral formulations, the percentages of BA, MP, and PP are stated to have maximum values of 2%, 0.18%, and 0.02% respectively [4]. While in oral dosage forms, their values should not exceed 3% for BA and 0.1–0.25% for both MP, and PP [4].

Due to the clinical impact of their quantitation, many articles in the literature were concerned with...
Figure 1. Structural formula of (A): benzyl alcohol, (B) methyl paraben, and (C) propyl paraben.

their analysis. Benzyl alcohol was analysed simultaneously with many active ingredients, as exemplified by its determination with granisetron in parenteral dosage forms [5], with vitamin A, D3, and E in veterinary oily injectable solution [6], with tolfenamic acid in veterinary pharmaceutical preparations [7], with benzaldehyde in injectable formulations [8], and with amcinonide in pharmaceutical preparations [9]. On the other hand, quantification of methyl and propyl parabens was the subject of concern of many articles whether with BA or with other preservatives in many matrices. From the most recent articles for their assay in cosmetics we mention their determination with other preservatives using capillary electrophoresis [10], micellar-electrokinetic chromatography [11] or liquid chromatography [12–14]. They were also determined with phthalates and other parabens using HPLC/DAD and GC-MS methods [15] and with antioxidants using supercritical fluid extraction combined with LC-MS [16]. GC-MS was also used for their determination following their derivatization with N, O-Bis (trimethylsilyl) acetamide [17] and they were also assayed in shampoo using liquid chromatography with amperometric detection [18].

Determination of parabens in food staff [19,20], pharmaceuticals [10,21,22], biological fluids [23–25], water samples [17,26], and environmental samples [27], has attracted attention of many researchers.

By reviewing the above-mentioned articles, it could be easily realized that HPLC was the most prevalent tool for the analysis of the concerned preservatives. Liquid chromatography is a powerful analysis tool to identify, separate and quantify various chemical compounds of diverse polarities [28]. The distribution constant $K_c$ – which describes the extent of the distribution of the analytes between the stationary phase and the mobile phase – determines the rate of elution of the studied species [28]. Separation efficiency is governed by many parameters like the retention factor, the selectivity factor, number and height of plates [28]. One of the most important applications of HPLC is its utility in chiral separations of racemic mixtures, where the pharmacological activity of racemic drugs is restricted to only one enantiomer, while the other may possess undesirable side effects or toxicological hazards [29–31]. These facts encouraged researchers to carry out chiral separation to quantify and resolve different enantiomers of pharmaceutical compounds belonging to different pharmacological classes using chiral columns, especially the polysaccharides based ones which proved to be highly efficient [29–31].

In this work, we present two different liquid chromatographic modes namely, gradient and isocratic elution, for the separation and quantitation of BA, MP, and PP in different pharmaceutical preparations.

Novelty of this work could be clearly demonstrated by presenting the first isocratic elution method to separate the three preservatives, which could be advantageous to laboratories that cannot operate the gradient mode especially in developing countries where consumption of such preservatives is relatively high, and consequently, their quantification is gaining attention to avoid the health hazards described above. In addition, the gradient mode succeeded to separate the preservatives from many active ingredients in the studied pharmaceuticals [diclofenac sodium, piroxicam, oxethazaine], which in turn offers a new method that could be applied for the assay of such pharmaceuticals. The gradient elution method was applied to determine the studied preservatives in different pharmaceutical preparations; moreover, full validation parameters were studied thoroughly for both modes.

2. Experimental

2.1. Instrumentation

Separations were performed using a Shimadzu SPD-20A model, equipped with 20 microlitre loop, SPD-20A UV/VIS. detector, CTO-20A column oven, DGU-207 degasser unit. Serial number: L 20135330505 AE,
220–230/240 V, 50–60 Hz, 160 VA, Kyoto, Japan. pH metre, Jenway, UK.

2.2. Materials and reagents

Benzyl alcohol (BA), Riedel de Haen, 99–100.5%, Germany.

Methyl 4-hydroxy benzoate (methyl paraben, MP) and Propyl 4-hydroxy benzoate (propyl paraben, MP), both of purity 99%, Sigma Aldrich, USA.

Methanol, HPLC grade, TEDIA, Fairfield, USA.

Acetonitrile and 2-propanol, HPLC grade, Fisher Scientific, UK.

Formic acid, ortho-phosphoric acid and acetone were all supplied as 85% minimum assay, anhydrous sodium sulphate (96%), diethyl ether and sulphuric acid were all provided by El Nasr Pharmaceutical Chemical Company, Egypt.

2.3. Pharmaceutical dosage forms

Laxolac® syrup, each 5 mL contains 3.35 gm lactulose. Batch # 160264. Medical Union Pharmaceuticals MUP, Egypt.

Elbavit® syrup, multivitamin and food supplement preparation. Batch # ML0240217. Multi-Apex for pharmaceutical industries-S.A.E, Egypt.

Mucogel® antacid suspension, contains 8.1 gm dried aluminium hydroxide gel, 2 gm magnesium hydroxide, 0.2 gm oxethazaine. Batch # 1609633. Egyptian International Pharmaceutical Industries Company E.I.P.I.C.O, Egypt.

Voltaren® ampoules, contains 75 mg/3 mL diclofenac natrium. Batch # Y1645. Novartis Pharma S.A.E. Egypt under license from Novartis Pharma A.G., Basle, Switzerland.

Feldene® ampoules, each 1 mL ampoule contains 20 mg piroxicam. Batch # 16022043. Manufactured by Global Pharmaceutical Industries for Pfizer Egypt.

Dispercam® ampoules, each 1 mL ampoule contains 20 mg piroxicam. Batch # 162494. Medical Union Pharmaceuticals MUP, Egypt.

Rheumarene® ampoules, each 3 mL ampoule contains 75 mg diclofenac sodium. Batch # 1014237. Produced by Pharco B International for South Egypt Drug Industries Company SEDICO.

Feldoral® ampoules, each 1 mL ampoule contains 20 mg piroxicam. Batch # 1015288/A. Produced by SEDICO Pharmaceutical Co., Egypt.

Salvatore Ferragamo®, body lotion, produced by Ferragamo Perfumes S.P.A., Firenze, Italy.

2.4. Chromatographic conditions

Separation was achieved on Prontosil Kromaplus C18 column, of dimension 250 x 4.6 mm, and particle size of 5 μm, Leonberg, Germany. Regarding gradient elution, two solvents were used, namely; acetonitril (solvent A), 0.1% formic acid of pH 2.9 (solvent B).

The gradient programme started with 40% solvent A and 60% for solvent B for the first 3 min, then 60% solvent A and 40% solvent B for the next 3 min, and finally equilibrating the system by returning back to the first ratio for the last 3 min.

Concerning the isocratic mode, a mobile phase composed of 0.1% formic acid of pH (2.9): 2-propanol in a ratio of 70:30 v/v was utilized. For both elution methods, the column temperature was set at 60°C, and the flow rate was 2 mL/min. Several steps were routinely followed prior to analysis including: ultra-filtration of the mobile phases using membrane filters, followed by sonication for at least 30 min. Besides, washing of the chromatographic system after analysis with methanol: double-distilled water – in a ratio of (1:1, v/v) – for 45 min was carried out.

2.5. Standard solutions and calibration

Both MP and PP stock solutions were prepared as 1.0 mg/mL in methanol, while BA was prepared as 10.0 mg/mL in the same solvent. The stock solutions were further diluted with methanol so that the working concentration ranges were (100.0–2000.0, 2.5–100.0 and 2.0–200.0 μg/mL) for BA, MP and PP respectively. Each concentration was injected three times to take the average values. The calibration curves were constructed by plotting peak area versus the final concentration in μg/mL.

2.6. Sample preparation

2.6.1. Liquid dosage forms

Liquid dosage forms were prepared by weighing accurately (5.0–10.0) gm in a separating funnel, then adding 2.5 mL of 10% sulphuric acid, followed by 10 mL of distilled water, and 20 mL of diethyl ether for extraction. The ether extract was washed with distilled water twice, each with 10 mL, after which, filtration over anhydrous sodium sulphate was done, eventually, evaporation on a water bath was carried out. The residue was dissolved in a minimum amount of methanol and quantitatively transferred into a 10.0-mL volumetric flask, and then the volume was completed to the mark with the same solvent.

2.6.2. Antacid suspension

Five grams of the suspension was accurately weighed into volumetric flask 50.0 mL, then 1 mL of o-phosphoric acid was added, followed by 20 mL of methanol. Acetone was then used to complete the volume to the mark. The prepared sample was then filtered and transferred quantitatively to another 50.0 mL volumetric flask, then the flask was made up to the volume with acetone.
2.6.3. Body lotion
One gram of the body lotion was accurately weighed in a beaker, then 15 mL of acetone was added, then the beaker was heated to dissolve the sample. Quantitative transfer of the solution to 50.0-mL volumetric flask was done using acetone to complete the volume to the mark. Then filtration was performed and the volume was completed to mark in another 50.0 mL volumetric flask.

All of the aforementioned procedures were followed according to an early published article [22].

2.6.4. Injectable solutions
Injectable solutions were simply diluted with methanol, so as to reach a final concentration of 10.0 mg/mL of BA. Serial dilution with methanol was performed to prepare different concentrations within the linearity range.

The specified concentrations of benzyl alcohol in different parental dosage forms were referred from the literature [8].

After extraction of different dosage forms, steps described under (Standard solutions and calibration) were performed and the concentrations of each preservative were determined from the corresponding regression equation.

3. Results
Both gradient and isocratic elution modes were proposed for the separation of BA, MP, and PP. In both modes, baseline separation was achieved accompanied with well-resolved peaks in a short chromatographic run (Figure 2). The proposed method allows quantification of the specified preservatives in their pure form, in addition to identifying their presence in different dosage forms followed by determining their concentrations. It is worth to mention that many pharmaceutical companies do not specify the type and concentration of the inactive ingredients added in the manufactured formulations; including preservatives. As a consequence, we referred to a published article [22] to identify some of the pharmaceutical formulations that contain MP and/or PP as a preservative and available in the local pharmacies. Furthermore, a reported method concerned with assay of BA [8] was used to determine the concentration of BA in different injectable preparations. To achieve optimum separation parameters accompanied with reasonable chromatographic run and best sensitivity, different experimental parameters were studied for both elution modes.

4. Discussion
4.1. Gradient liquid chromatography
Several trials were attempted to select the optimum programme timing, which depends mainly on the retention times of the studied preservatives. Different organic modifiers were investigated such as methanol, 1-propanol, and acetonitril. Although methanol succeeded to separate the three peaks efficiently, it resulted in a delay in the retention times. Upon using 1-propanol overlap between the peaks of BA and MP occurred. Acetonitril on the other hand separated the three peaks effectively and reduced the elution times, so it was selected for the study.

The ratio of solvent A (acetonitril) to solvent B (0.1% formic acid) was also investigated over the range of 10:90 to 90:10; v/v. Changing the ratio significantly affected the separation efficiency. The different ratios studied with the accompanied programme timing are listed in Table 1. From this table it could be realized that the last two ratios were very close in their results, since they achieved best separation in a shorter time, however, the last ratio was preferred as it resulted in slightly better separation parameters.

The column temperature was a major factor in the resolution. When the system was operated at ambient temperature, overlap between the peaks of BA and MP takes place. So, different column heating temperatures were attempted starting from 30°C to 60°C. It was found that 60°C was the appropriate choice as it resulted in successful separation between BA and MP with high repeatability as well as reduction in the retention times of the three peaks. The flow rate was also changed over the range 1–2.5 mL/min, where 2 mL/min was chosen as it shortens the chromatographic run without affecting the peaks symmetry.

To obtain the highest sensitivity, different wavelength settings were tested; starting from 210 nm up to 260 nm. After detailed investigation, 254 nm was selected and applied through the study as it gives high peak areas for the three studied preservatives accompanied with acceptable base line separation which was
Table 1. Comparison between different gradient programmes attempted for optimization.

| Solvent A | Solvent B | Programme timing | Preservative | Retention times | Resolution | K' | Tailing factor | NTP |
|-----------|-----------|------------------|--------------|----------------|------------|----|---------------|-----|
| methanol  | 0.1% formic acid | 40:60, v/v for the first 20 min, 60:40, v/v for the next 5 min, return for first ratio for 10 min. | BA          | 9.566         | –           | –  | 0.960         | 7295 |
|           |            |                  | MP          | 15.063        | 9.542      | 0.575 | 0.897         | 7320 |
|           |            |                  | PP          | 23.834        | 16.377     | 1.492 | 1.012         | 67,518 |
| 1-propanol| 0.1% formic acid | 40:60, v/v for the first 5 min, 60:40, v/v for the next 5 min, return for first ratio for 5 min. | BA & MP     | 3.770*        | 0.210      | 0.679 | 1.167         | 4985 |
|           |            |                  | PP          | 6.987         | 3.465      | 2.111 | 1.083         | 1943 |
| acetonitril| 0.1% formic acid | 10:90, v/v for the first 10 min, 90:10, v/v for the next 10 min, return for first ratio for 10 min. | BA          | 4.807         | 2.631      | 11.106| 1.093         | 11,473 |
|           |            |                  | MP          | 8.733         | 1.771      | 20.993| 1.334         | 2223 |
|           |            |                  | PP          | 17.061        | 0.266      | 41.967| 1.212         | 318,548 |
| acetonitril| 0.1% formic acid | 20:80, v/v for the first 10 min, 80:20, v/v for the next 10 min, return for first ratio for 10 min. | BA          | 4.964         | 2.264      | 9.646 | 1.167         | 4985 |
|           |            |                  | MP          | 7.971         | 4.566      | 16.095| 1.089         | 794  |
|           |            |                  | PP          | 17.236        | 0.314      | 35.963| 1.167         | 288,537 |
| acetonitril| 0.1% formic acid | 30:70, v/v for the first 3 min, 70:30, v/v for the next 3 min, return for first ratio for 3 min. | BA          | 2.171         | 0.408      | 0.943 | 1.201         | 6230 |
|           |            |                  | MP          | 2.377         | 1.738      | 1.124 | 1.151         | 6004 |
|           |            |                  | PP          | 5.131         | 17.492     | 3.550 | 1.051         | 11,766 |
| acetonitril| 0.1% formic acid | 40:60, v/v for the first 3 min, 60:40, v/v for the next 3 min, return for first ratio for 3 min. | BA          | 2.177         | 1.893      | 1.186 | 1.205         | 6117 |
|           |            |                  | MP          | 2.398         | 1.195      | 3.785 | 1.139         | 6099 |
|           |            |                  | PP          | 5.250         | 18.252     | 4.948 | 1.042         | 12,262 |

*BA and MP eluted as one single peak.

not achieved in other lower wavelength settings that showed better sensitivity than 254 nm.

4.2. Isocratic liquid chromatography

By reviewing the literature, it could be recognized that the studied preservatives were not successfully separated before by isocratic liquid chromatography, which gives us the enthusiasm to introduce this mode for the first time which is advantageous to many laboratories where gradient mode is not accessible, especially in many quality control laboratories in the developing countries. After detailed investigation, it was concluded that the type and ratio of organic modifier is the key element to achieve isocratic separation.

Methanol, acetonitril, and 1-propanol were all tried at different ratios, but none of them succeeded to elute PP, which was retained on the column for more than 40 min, besides, in many used ratios, peaks of BA and MP overlapped. Only 2-propanol eluted PP from the column, keeping in consideration that its ratio is very critical to separate BA from MP. The ratio of 0.1% formic acid: 2-propanol was modified to achieve well-separated peaks, after several experimental trials, it was found that only a ratio of 70:30, v/v was suitable to reach this target. When a ratio of 50:50 v/v, or 60:40 v/v for the two solvents respectively was used, BA and MP overlapped, and were eluted in the form of one single peak. It is to be mentioned that we kept the column temperature at 60°C and the flow rate at 2 mL/min, with UV detection at 254 nm as described in the previous section for the same reasons. In addition, heating the column helped to decrease the high pressure obtained upon using 2-propanol as an organic modifier which could be attributed to its high viscosity, thus maintaining the chromatographic stability of the system.

A summary of the separation parameters at different experimental conditions for the two elution modes is abridged in Table 2.

By comparing the results obtained from the proposed method – with both modes of separation – with previously published articles, the proposed method was superior in many aspects. Regarding analysis time, both gradient and isocratic modes revealed shorter retention times for all analytes than those obtained from reference methods [5,14,15,19,21] (Table 3). Moreover, the proposed methods showed wider linearity ranges for the studied preservatives than the comparison methods [5–9,12,14,19,21] (Table 3). Furthermore, separation of BA using the proposed methods consumed lower ratios of organic solvents than some of the published methods [7,9], where utility of high percentages of organic solvents – that may reach a value of 70% was essential for the separation. Such advantage is in accordance with application of green chemistry in pharmaceutical analysis supporting the eco-friendly analytical methods, and reducing harmful effects on analysts.

4.3. Method validation

According to Harris [32], method validation is conducted to prove that the proposed method is acceptable for its intended purpose. Accordingly, method validation for both elution modes was carried out according to USP guidelines [1].

Linearity; which measures that the response is proportional to the quantity of the analyte, was studied using the described chromatographic conditions, where a linear relationship was established by plotting the peak areas versus the concentrations in μg/mL. For both gradient, and isocratic modes, the linear concentration ranges of BA, MP, and PP were 100.0–2000.0,
Table 2. Summary of separation parameters during optimization of the proposed method.

| Parameter                  | Preservative | Retention times | Resolution | K' | Tailing factor | NTP |
|----------------------------|--------------|-----------------|------------|----|---------------|-----|
| Gradient mode              |              |                 |            |    |               |     |
| 30°C                       |              |                 |            |    |               |     |
| BA&MP                      | 9.246        | 13.297          | 4.845      | 1.061 | 51.12         |     |
| PP                         | 15.833       | 3.912           | 9.010      | 2.395 | 494           |     |
| 40°C                       |              |                 |            |    |               |     |
| BA                         | 4.525        | 3.796           | 1.362      | 1.070 | 11.402        |     |
| MP                         | 5.224        | 22.851          | 4.380      | 1.027 | 11.064        |     |
| PP                         | 11.897       | 2.859           | 6.140      | 1.722 | 15.247        |     |
| 50°C                       |              |                 |            |    |               |     |
| BA                         | 4.549        | 1.899           | 1.026      | 1.076 | 10.874        |     |
| MP                         | 5.189        | 3.362           | 1.311      | 1.044 | 10.136        |     |
| PP                         | 11.797       | 15.673          | 4.255      | 1.011 | 54.060        |     |
| 60°C                       |              |                 |            |    |               |     |
| BA                         | 4.310        | 3.108           | 0.953      | 1.074 | 10.380        |     |
| MP                         | 4.742        | 2.371           | 1.149      | 1.036 | 9.420         |     |
| PP                         | 10.284       | 10.242          | 3.660      | 1.016 | 14.911        |     |
| 2.5–100.0, and 2.0–200.0 μg/mL respectively. Calculation of the square of the correlation coefficient $R^2$ was carried out, and it was found that its value in all the cases approaches unity demonstrating low scattering of the points around the calibration graphs and emphasizing linearity.

Regression equations for BA using isocratic and gradient elution modes respectively are: $Y = -7 + 236X$, $Y = -1 + 236X$. |
Table 3. Comparison between the obtained retention times and linearity ranges of the studied preservatives using the proposed and comparison methods.

| Parameter                  | Studied preservative | Proposed methods | Reference methods results | Reference methods |
|----------------------------|----------------------|------------------|---------------------------|-------------------|
| Retention time (min)       | BA                   | 2.177            | 4.024                     | 6                 |
|                            |                      |                  |                           | [5]               |
|                            | MP                   | 2.398            | 4.909                     | 7.2               |
|                            |                      |                  |                           | [14]              |
|                            | PP                   | 5.250            | 7.783                     | 10.95             |
|                            |                      |                  |                           | [14]              |
| Linearity range (μg/mL)    | BA                   | 100.0–2000.0     | 100.0–300.0               | 1250.0–5000.0     |
|                            |                      |                  |                           | [6]               |
|                            |                      |                  |                           | 400.0–600.0       |
|                            |                      |                  |                           | [7]               |
|                            |                      |                  |                           | 10.0–100.0        |
|                            |                      |                  |                           | [8]               |
|                            |                      |                  |                           | 60.0–600.0        |
|                            |                      |                  |                           | [9]               |
|                            |                      |                  |                           | 15.0–100.0        |
|                            |                      |                  |                           | [12]              |
|                            |                      |                  |                           | 75.0–450.0        |
|                            | MP                   | 2.5–100.0        | 0.25–10.0                 | 0.5–45.0          |
|                            |                      |                  |                           | [12]              |
|                            | PP                   | 2.0–200.0        | 0.5–6.0                   | 1.0–75.0          |
|                            |                      |                  |                           | [14]              |
|                            |                      |                  |                           | 1.0–3.0           |

Y = −33 + 244X, corresponding equations for MP are: Y = −110 + 411X, Y = −320 + 345X, and eventually for PP, the regression equations are: Y = −120 + 366X, Y = −230 + 273X.

The limit of detection (LOD) was practically determined according to USP [1] as the lowest concentration that gives a signal to noise ratio of 3:1. Using gradient elution, LOD for BA, MP, and PP were found to be 50.0, 1.8, and 1.5 μg/mL respectively. The corresponding values in isocratic elution were 60.0, 1.5, and 1.3 μg/mL. Similarly, the limit of quantitation (LOQ) was experimentally determined so as to accomplish 10:1 ratio for signal to noise. The determined values for BA, MP, and PP using gradient mode are 70.0, 2.0, and 1.8 μg/mL respectively. On the other hand, isocratic elution resulted in corresponding values of 80.0, 2.3, and 1.7 μg/mL.

Furthermore, the accuracy of the proposed method was investigated by analysing samples of the pure powder of the preservatives over the working concentration ranges. The high percentage found values indicate the closeness of the results to the true values (Table 4). The results obtained were compared with the reference method [14] and they were found to be in good agreement as indicated by the small values of student t-test and variance ratio F test [33].

4.4. Analysis of pharmaceuticals

The proposed gradient liquid chromatographic method was applied to assay BA in different injectable solutions, besides its utilization to detect and quantify MP and/or PP in various oral liquid dosage forms (Figure 3 and Table 6). By reviewing Table 6, it could be deduced that the concentrations of all three preservatives are within the allowed values [4]. Besides, it is clear from the chromatograms under Figure 3 concerned with assay of BA, that many active ingredients could be well separated using this mode of elution under the described chromatographic conditions, from which we mention: diclofenac sodium that appear at 7.86 min (Figure 3(E,F)), and piroxicam which is eluted at 4.38 min (Figure 3(G–I)). For both drugs well-separated peaks are observed which gives the opportunity for researchers to use the proposed method for their analysis. Regarding formulations assayed to quantify MP and PP, clear baseline separation and well-resolved peaks of the active ingredients are also obtained. Figure 3(C) shows oxethazaine which has a retention time of 3.38 min. While in some formulations, more than one peak appears, and it was puzzling to determine which one represent the active ingredient (Figure 3(A)), these peaks might represent other inactive ingredients in the formulations. The other case was the appearance of peaks, but since the formulation itself has multiple ingredients, it was challenging to specify which ingredient is presented by such peaks; this was demonstrated in Figure 3(B) of Elbavit® multivitamin syrup extract, and Figure 3(D) presenting Salvatore Ferragamo® body lotion extract.
Table 4. Determination of the three preservatives in pure form using the proposed method.

| Parameter      | Taken (μg/mL) | Gradient elution | Isocratic elution | Reference method [14], % found |
|----------------|---------------|------------------|-------------------|-------------------------------|
|                | Found (μg/mL) | % Found          | Found (μg/mL)     | % Found                       |
| Benzyl alcohol | 100.0         | 98.25            | 98.25             | 98.75                         | 98.75                        | 99.58 |
|                | 200.0         | 199.04           | 99.52             | 195.76                        | 97.88                        | 98.45 |
|                | 400.0         | 401.80           | 100.45            | 402.32                        | 100.58                       | 100.45 |
|                | 600.0         | 603.48           | 100.58            | 609.48                        | 101.58                       | 100.25 |
|                | 1000.0        | 1009.5           | 100.95            | 1022.3                        | 100.23                       | 100.25 |
|                | 1300.0        | 1298.11          | 99.87             | 1311.57                       | 100.38                       | 100.25 |
|                | 1600.0        | 1578.4           | 98.65             | 1592.64                       | 99.54                        | 100.58 |
|                | 2000.0        | 1982.4           | 99.12             | 1977.8                        | 98.89                        | 100.25 |
| Mean ±SD       |               | 99.67 ± 0.96     |                   | 99.79 ± 1.24                  | 99.68 ± 0.91                 |
| t test         |               | 0.37             |                   | 0.76                          |                              |
| F test         |               | 1.1              |                   | 1.86                          |                              |
| Methyl paraben | 2.5           | 2.46             | 98.52             | 2.49                          | 99.52                        | 99.52 |
|                | 10.0          | 9.96             | 99.58             | 9.96                          | 99.64                        | 99.78 |
|                | 20.0          | 19.96            | 99.78             | 20.09                         | 100.48                       | 100.47 |
|                | 30.0          | 30.26            | 100.85            | 30.17                         | 100.58                       | 101.52 |
|                | 50.0          | 50.46            | 100.92            | 50.63                         | 101.25                       |          |
|                | 60.0          | 60.77            | 101.28            | 60.99                         | 101.65                       |          |
|                | 80.0          | 81.25            | 101.56            | 80.18                         | 100.22                       |          |
|                | 100.0         | 100.88           | 99.83             | 99.83                         |                              |          |
| Mean ±SD       |               | 99.42 ± 1.03     |                   | 100.39 ± 0.76                 | 100.33 ± 0.89                |
| t test         |               | 0.04             |                   | 0.14                          |                              |
| F test         |               | 1.34             |                   | 1.37                          |                              |
| Propyl paraben | 2.0           | 1.98             | 99.05             | 1.99                          | 99.52                        | 100.52 |
|                | 10.0          | 10.03            | 100.25            | 9.88                          | 98.78                        | 99.65 |
|                | 30.0          | 29.67            | 98.89             | 29.91                         | 99.49                        | 99.89 |
|                | 70.0          | 69.13            | 98.75             | 70.61                         | 100.87                       | 100.77 |
|                | 100.0         | 100.65           | 100.65            | 100.47                        | 100.47                       |          |
|                | 120.0         | 120.56           | 100.47            | 120.14                        | 100.12                       |          |
|                | 150.0         | 151.17           | 100.78            | 149.96                        | 99.97                        |          |
|                | 200.0         | 199.92           | 99.96             | 199.1                         | 99.55                        |          |
| Mean ±SD       |               | 99.85 ± 0.83     |                   | 99.87 ± 0.64                  | 99.96 ± 0.86                 |
| t test         |               | 0.56             |                   | 0.96                          |                              |
| F test         |               | 1.07             |                   | 1.8                           |                              |

Each result is the average of three determinations. 2.365 and 4.35 are tabulated t and F values at \( P = 0.05 \) [33].

Table 5. Precision data of the proposed method.

| Parameter      | Taken (μg/mL) | Gradient elution | Isocratic elution | Intermediate precision |
|----------------|---------------|------------------|-------------------|------------------------|
|                | Found (μg/mL) | % Found          | Found (μg/mL)     | % Found                |
| Benzyl alcohol | 100.0         | 100.58           | 99.65             | 99.05                  | 99.87                       |
|                | 1000.0        | 100.78           | 99.78             | 98.78                  | 99.56                       |
|                | 2000.0        | 99.78            | 100.89            | 98.45                  | 98.78                       |
| Mean ±SD       |               | 100.38 ± 0.53    | 100.11 ± 0.68     | 98.76 ± 0.31           | 99.4 ± 0.56                  |
| Methyl paraben | 2.5           | 100.58           | 99.58             | 99.78                  | 99.32                       |
|                | 50.0          | 99.65            | 99.45             | 98.85                  | 99.14                       |
|                | 100.0         | 99.12            | 100.45            | 99.61                  | 99.14                       |
| Mean ±SD       |               | 99.78 ± 0.74     | 99.83 ± 0.54      | 99.41 ± 0.49           | 98.97 ± 0.46                  |
| Propyl paraben | 2.0           | 100.45           | 101.58            | 99.45                  | 100.45                       |
|                | 100.0         | 100.03           | 100.45            | 99.05                  | 100.23                       |
|                | 200.0         | 101.25           | 99.65             | 99.25                  | 99.32                       |
| Mean ±SD       |               | 99.87 ± 0.68     | 100.56 ± 0.97     | 99.92 ± 1.17           | 100.05 ± 0.59                |

5. Comparative perspective

Both of the proposed gradient and isocratic liquid chromatographic modes could separate BA, MP, and PP successfully. In spite that the gradient mode has a shorter chromatographic run than the isocratic mode (Figure 2), the latter showed several advantages like: better separation parameters (Table 2), being more versatile and widespread in quality control laboratories, and environmentally benign since lower percentages of organic solvent are consumed during analysis.

By comparing the validation parameters, it could be noticed that gradient elution showed high specificity since it could distinguish the concerned preservatives from other matrices in the sample. This could be well demonstrated from the analysis of different dosage forms where the peaks of different active ingredients did not interfere with the peaks of BA, MP, or PP (Figure 3). This advantage makes this mode a candidate for separation and quantification of various pharmaceutical compounds which were studied in this work, using the prescribed chromatographic conditions, which in turn encourages researchers to quantify such drugs in their pharmaceuticals.

On the contrary, significant overlap of different active ingredients with the peaks of the preservatives
Table 6. Application of the proposed method for the analysis of different dosage forms.

| Dosage form                          | Concentration (gm%) |
|--------------------------------------|---------------------|
|                                      | BA      | MP      | PP     |
| Laxolac® syrup (each 5 mL contains 3.35 gm lactulose. Batch # 160264, Medical Union Pharmaceuticals MUP, Egypt) | 0.0464  | 0.016   | –      |
| Elbavit® syrup (multivitamin and food supplement preparation. Batch # ML0240217. Multi-Apex for pharmaceutical industries-S.A.E, Egypt) | –      | 0.00996 | –      |
| Mucogel® antacid suspension (8.1 gm dried aluminium hydroxide gel, 2 gm magnesium hydroxide, 0.2 gm oxethazaine. Batch # 1609633. Egyptian International Pharmaceutical Industries Company E.I.P.I.C.O, Egypt) | –      | 0.066   | –      |
| Salvatore Ferragamo®, body lotion (produced by Ferragamo Perfumes S.P.A., Firenze, Italy) | –      | 0.685   | 0.375  |

Parenteral preparations containing benzyl alcohol

|                                    | Taken (µg/mL) | Found | % Founda | Reference method [14], % found |
|------------------------------------|---------------|-------|----------|-------------------------------|
| Rheumarene® ampoules (contains 75 mg diclofenac sodium. Batch # 1014237. Produced by Pharco B International for South Egypt Drug Industries Company SEDICO) | 100.0  | 100.25 | 100.25   | 100.25                        |
|                                    | 300.0         | 299.61| 99.87    | 99.56                        |
|                                    | 600.0         | 596.76| 99.46    | 99.45                        |
|                                    | 1000.0        | 990.5 | 99.05    |                               |
| Mean ±SD                           | 99.66±0.52    |       |          | 99.75±0.43                   |
| t test                             | 0.24          |       |          |                               |
| F test                             | 1.46          |       |          |                               |
| Voltaren® ampoules (contains 75 mg/3 mL diclofenac sodium. Batch # Y1645. Novartis Pharma S.A.E. Egypt) | 100.0  | 100.58 | 100.58   | 99.58                        |
|                                    | 300.0         | 304.74| 101.58   | 99.12                        |
|                                    | 600.0         | 599.7 | 99.95    | 101.52                       |
|                                    | 1000.0        | 100.66| 100.66   |                               |
| Mean ±SD                           | 100.69±0.67   |       |          | 100.07±1.27                  |
| t test                             | 0.39          |       |          |                               |
| F test                             | 3.59          |       |          |                               |
| Feldene® ampoules (contains 20 mg piroxicam. Batch # 16022043. Manufactured by Global Pharmaceutical Industries for Pfizer Egypt) | 300.0  | 301.35 | 100.45   | 101.58                       |
|                                    | 500.0         | 506.25| 101.25   | 100.36                       |
|                                    | 800.0         | 797.52| 99.69    | 99.89                        |
|                                    | 1000.0        | 998.5 | 99.85    |                               |
| Mean ±SD                           | 100.31±0.71   |       |          | 100.61±0.87                  |
| t test                             | 0.68          |       |          |                               |
| F test                             | 1.5           |       |          |                               |
| Feldoral® ampoules (contains 20 mg piroxicam. Batch # 1015288/A. Produced by SEDICO Pharmaceutical Co., Egypt) | 100.0  | 100.45 | 100.45   | 100.45                       |
|                                    | 500.0         | 506.25| 101.25   | 100.85                       |
|                                    | 700.0         | 694.75| 99.25    | 99.69                        |
|                                    | 1000.0        | 996.9 | 99.69    |                               |
| Mean ±SD                           | 100.16±1.088  |       |          | 100.33±0.59                  |
| t test                             | 0.88          |       |          |                               |
| F test                             | 2.22          |       |          |                               |
| Dispercam® ampoules (contains 20 mg piroxicam. Batch # 162494. Medical Union Pharmaceuticals MUP, Egypt) | 200.0  | 201.56 | 100.78   | 100.77                       |
|                                    | 400.0         | 403.92| 100.98   | 100.69                       |
|                                    | 700.0         | 699.23| 99.89    | 101.45                       |
|                                    | 1000.0        | 993.5 | 99.35    |                               |
| Mean ±SD                           | 100.25±0.76   |       |          | 100.97±0.42                  |
| t test                             | 0.85          |       |          |                               |
| F test                             | 3.27          |       |          |                               |

aEach result is the average of three determinations.

b3.182 and 9.55 are tabulated t and F values at P = 0.05 [33].

Under investigation resulted when isocratic elution was applied, hence, quantification of BA, MP, and PP in various pharmaceuticals was performed using gradient mode.

Regarding robustness, the gradient mode was superior to isocratic one. As described before, the minor changes in the ratio of 0.1% formic acid to acetonitrile in the gradient mode, did not affect separation parameters greatly (Table 1), even if methanol was used as organic modifier, well separation of the three peaks takes place, in spite of the delayed retention times. On the other hand, isocratic elution suffered from poor robustness. Separation of the three preservatives couldn’t be achieved if any other solvent than 2-propanol was used. Besides, if the ratio of 0.1% formic acid: 2-propanol was deliberately changed, overlap of the peaks of BA and MP takes place. It is worth to mention that both modes proved to be linear over the working concentration ranges, accurate and precise.
6. Conclusion

The proposed method succeeded to separate BA, MP, and PP using gradient and isocratic liquid chromatographic modes, which in turn were fully validated. Gradient elution was applied to assay the three preservatives in different pharmaceuticals showing that the calculated amounts are within the stated ranges, besides, it was possible to separate many active ingredients in the studied formulations which imparts versatility for the gradient elution mode using the described chromatographic conditions. Gradient elution showed shorter chromatographic runs than isocratic mode, while the latter demonstrated better separation parameters and consumed limited amounts of organic solvents; eco-friendly. Gradient elution was found to be superior over isocratic mode in some validation parameters like being more specific and showing acceptable robustness.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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