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Research Article

Determination of the design space of the HPLC analysis of water-soluble vitamins

Analysis of water-soluble vitamins has been tremendously approached through the last decades. A multitude of HPLC methods have been reported with a variety of advantages/shortcomings, yet, the design space of HPLC analysis of these vitamins was not defined in any of these reports. As per the food and drug administration (FDA), implementing the quality by design approach for the analysis of commercially available mixtures is hypothesized to enhance the pharmaceutical industry via facilitating the process of analytical method development and approval. This work illustrates a multifactorial optimization of three measured plus seven calculated influential HPLC parameters on the analysis of a mixture containing seven common water-soluble vitamins (B_1, B_2, B_6, B_{12}, C, PABA, and PP). These three measured parameters are gradient time, temperature, and ternary eluent composition (B1/B2) and the seven calculated parameters are flow rate, column length, column internal diameter, dwell volume, extracolumn volume, %B (start), and %B (end). The design is based on 12 experiments in which, examining of the multifactorial effects of these 3 + 7 parameters on the critical resolution and selectivity, was carried out by systematical variation of all these parameters simultaneously. The 12 basic runs were based on two different gradient time each at two different temperatures, repeated at three different ternary eluent compositions (methanol or acetonitrile or a mixture of both). Multidimensional robust regions of high critical R_s were defined and graphically verified. The optimum method was selected based on the best resolution separation in the shortest run time for a synthetic mixture, followed by application on two pharmaceutical preparations available in the market. The predicted retention times of all peaks were found to be in good match with the virtual ones. In conclusion, the presented report offers an accurate determination of the design space for critical resolution in the analysis of water-soluble vitamins by HPLC, which would help the regulatory authorities to judge the validity of presented analytical methods for approval.

Keywords: 3D-Modeling software / Method development / Quality by design (QbD) / Water-soluble vitamins
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1 Introduction

In order to develop a method in the past, "the trial and error approach" was commonly used, until a satisfactory separation was achieved. The complexity of matrices, variability in drug nature, cost, and time factors were all rate-limiting steps even for an experienced chromatographer. Therefore, computer-simulation softwares and in silico methods for optimization of HPLC analytical methods were sometimes used as they offer the right tool for these predictions, where a limited number of "real" experiments is required for the development of a huge number of predicted runs. Computer-assisted design in method development has received much attention within the pharmaceutical industry, mainly from the perspectives of cost savings associated with faster and more efficient development, not to mention that implementation of the quality by design (QbD) starts to be a requirement by regulatory authorities food and drug administration (FDA) to advance product and process quality in the industry as a vehicle for the transformation of how drugs are discovered, developed, analyzed, and commercially manufactured. QbD is defined by the FDA as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management", as described in the International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (Q8R1) [1].

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Abbreviations: **PABA**, *para*-aminobenzoic acid; **QbD**, quality by design

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The concept of the QbD was mentioned also in the International Conference on Harmonization (Q8R2) guidance. It stated that "Quality cannot be tested into products; it has to be built in by design". QbD concepts and terminology need to be applied for analytical methods in order to develop robust method and allow for smooth analytical method transfer for the same product in different laboratories and in different countries.

It will transform the chemistry, manufacturing, and controls regulatory review into a modern science-based pharmaceutical quality control [2].

One of the most widely used softwares in defining an HPLC method design space nowadays is DryLab® [3–11]. The software predicted resolution maps comprise mathematical calculations related to basic work of Snyder, Dolan, and Molnár and as well on the solvophobic theory, which has been studied and explained in details by Horváth, Melander, and Molnár decades ago [12–14]. On the basis of two or more real runs for a given mixture, the program can predict the effect of varying other parameters such as mobile phase composition, flow rate, temperature, isocratic or gradient modes, or change in stationary phase conditions (column dimensions, particle size), The resolution map obtained by the software can be 1D: allowing variation of only one factor at a time (for example, t_G, %B, pH, etc.), or 2D: allowing varying two variables simultaneously (for examples, gradient time (t_G) versus pH, percentage organic in the mobile phase (%B) versus pH, t_G versus temperature (T), ionic strength versus T, among many others possible combinations). A new extension of the 2D mode is the recently commercially available "Cube" which shows a 3D resolution map.

The "cube" is a modern approach to HPLC method development within the QbD framework. It is an advanced resolution and selectivity mapping technology [15]. It is based on only 12 experimental input runs carried out by systematically varying all factors previously mentioned simultaneously. These runs build the "resolution cube", from which the chromatographer can investigate more than one million virtual chromatograms with extremely high precision and can evaluate the effects of t_G, T, and ternary composition to find the best separation conditions, and identification of the peak pair with critical resolution ($R_{s,crit}$) among all the peaks of the chromatogram. The 3D modeling offers visual support of the design space that generates more flexibility and establishes more robust HPLC regions for utilization [16]. The potential of this multifactorial modeling will be in cost reduction, generation of robust methods, smoother method transfer, and also the replacement of acetonitrile by methanol which is very critical due to the inflated price of acetonitrile and the tendency toward greener solvents [15].

Several papers have been published on the application of the 3D modeling resolution map of DryLab® for development of HPLC methods [15–17], targeting commercially available pharmaceutical preparations as this approach to HPLC method development is extremely beneficial to the industry and would represent a plus at the authorities responsible for pharmaceutical product approval for release to the market.

On the other hand, water-soluble vitamins are a broad group of organic compounds required to maintain normal cellular and metabolic functions of human and animal body. Many combinations of these vitamins are available in the market worldwide that explains the enormous number of RP-HPLC methods reported in the literature for water-soluble vitamins [18–23]. None of these methods defines the exact design space of their separation by HPLC. Hence, the aim of this work is to extend the application of the 3D modeling resolution map to define the design space of water-soluble vitamins separation for the first time and application of the resulting information on vitamin mixtures, which are commercially available in the market achieving the maximum possible resolution between all compounds with a minimum run time, all within a known robust working space.

2 Materials and methods

2.1 Eluents

Methanol and acetonitrile (HPLC grade). Monobasic sodium monophosphate (NaH_2PO_4) and phosphoric acid (H_3PO_4) (Sigma-Aldrich, Germany).

Eluent A was 25 mM phosphate buffer pH 2.60. It was prepared by combining varying volumes of aqueous buffers A_1 and A_2, where A_1 is a solution 0.1 M phosphoric acid and A_2 is a solution 0.1 M monobasic sodium monophosphate.

Eluent B was varied between acetonitrile (B_1) and methanol (B_2). From 5 to 100% B gradient elution was used at a flow rate of 1 mL/min in two gradient times 20 min and 60 min, each at two different temperatures of 30 and 60°C. Ternary eluents were acetonitrile (100%), methanol (100%), and a mixture of both (acetonitrile/methanol) (50:50, v:v).

2.2 The injection solutions

2.2.1 The laboratory prepared standard mixture solution

It was freshly prepared every day due to the instability of ascorbic acid by dissolving 0.1 mg/mL of thiamin (B1), ascorbic acid (C), nicotinamide (PP), *para*-aminobenzoic acid (PABA), and riboflavin (B2) in 25 mM phosphate buffer while pyridoxine (B6) and cyanocobalamine (B12) were prepared in 1 mg/mL-solutions due to the low molar absorptivity at the wavelength of determination. All reference standards were kindly supplied by SEDICO, Egypt.

2.2.2 The Pharmaceutical preparation

Royal Vit G capsules® (SEDICO, Egypt): It contains vitamins B1, PP, PABA, B2, B12, B6, B5, A, E, D3; manganese sulphate; biotin; lysine; ginseng extract; dimethylaminoethanol bitartrate; bee pollen powder; bees wax, dicalcium hydrogen phosphate; ferrous fumarate; folic acid; and wheat germ oil.

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The capsule content was dissolved in 25 mL 25 mM phosphate buffer (pH 2.60). Prior to injection, the sample was filtered through 0.45-μm membrane filter.

Sport Drink Effervescent Instant® (SEDICO, Egypt): It contains vitamins B1, PP, C, B2, B6, B12, B5; biotin; dicalcium hydrogen phosphate; and folic acid. The powder was dissolved in 100 mL 25 mM phosphate buffer (pH 2.60). Prior to injection, the sample was filtered through 0.45-μm membrane filter.

2.3 Equipments

HPLC separations were performed Thermo Finnigan Spectrasystem® (UK) that consists of pump P2000, detector UV3000, autosampler AS3000, and data acquisition software for HPLC Chromquest 4.2 data system (UK). UV detection was performed at 254 nm. The dwell volume was 2.03 mL. Hypersil Gold C18 column, (250 × 4.6 mm, 3 μm) was purchased from Thermo Scientific, Germany.

2.4 Modeling software

DryLab®2010, which includes PeakMatch® v. 3.60 and DryLab® v.3.95 (Molnár-Institute, Berlin, Germany) was used. Peaks were identified and aligned based on peak areas using the user friendly tools of PeakMatch®, such as peak turnover and peak splitting functions of the software, reducing the usual problems of common misalignments between peaks. Modeling was performed in DryLab®2010 and predictions were compared with the original experiments to control the validity of the modeling process. Generation of 3D resolution models was carried out with a new proprietary algorithm in DryLab®2010.

2.5 Methods

Four initial input data runs were acquired with gradient times (t_G) of 20 and 60 min, at temperatures of 30 and 60°C. The four input runs were repeated three times at different eluent B1/B2 ternary compositions, t_C (a) 100% acetonitrile, (b) acetonitrile/methanol (50:50, v:v), and (c) 100% methanol. Repetitive injections of the sample mixture were performed for each set-up, until at least two consecutive reproducible chromatograms were obtained regarding peak areas (±10%) and retention times (±0.02 min). Peak areas are of crucial importance, as they represent the tool for identification of the sample components, when injected in different chromatographic conditions. Peak areas do not significantly change as long as the same injection volume of the same mixture solution at the same flow rate is applied.

After the 12 runs (i.e., four runs × three times with different ternary eluents) were finished, they were exported to PeakMatch® for the peak tracking process, then data were transferred to DryLab®2010.

3 Results and discussion

The seven water-soluble vitamins under investigation were B_1, C, PP, B_6, PABA, B_{12}, and B_2. The pharmaceutical preparations contain other water-soluble compounds such as manganese sulphate, biotin, lysine, B_5, ginseng extract, and dimethylaminoethanol bitartrate. Each of these compounds was injected at $t_G = 20$ min and $T = 30$°C, it was found that it was not detectable at the working wavelength (254 nm). The pharmaceutical preparations contains also fat-soluble vitamins such as A, E, and D_3, as well as other water-insoluble substances such as bee pollen powder, bees wax, dicalcium hydrogen phosphate, ferrous fumarate, folic acid, and wheat germ oil. The fat-soluble material was discarded after dissolving the pharmaceutical preparation in the aqueous phase and filtration.

3.1 Basic runs

The systematic design of experiments of the 3D modeling involved 12 basic runs. Eluent A was 25 mM phosphate buffer pH 2.60 in all the basic runs. As higher pH was tried, it was found, that, a minor prolongation in the retention time of the first four peaks (B1, C, PP, and B6) occurred with some resolution decrease; however, no significant effect was observed on the last three peaks (PABA, B12, and B2). Hence, pH 2.60 was selected. The study of the effect of ternary eluent was meant to reduce the amount of acetonitrile in the mobile phase due to its actual shortage and its high price. The retention time of each of the seven water-soluble vitamins at the 12 basic runs are listed in Table 1.

3.1.1 Runs where eluent B was methanol only

It was found that at t_G20_T60°C, the retention time of all analytes were shortened by increasing the temperature and resolution of PP and B6 was decreased, in comparison of that at t_G20_T30°C. The "flat" gradient (60 min) had almost no effect on the retention of the first four peaks with slight prolongation in retention the last three peaks.

3.1.2 Runs where eluent B was methanol/acetonitrile (50:50, v/v)

Retention times of all peaks have decreased compared to runs in which the ternary eluent was methanol only due to the stronger eluent strength of acetonitrile than methanol.

At t_G20_T30°C vitamin C almost coeluted with PP, while at t_G20_T60°C, other than shortening in retention times associated with temperature increase, better resolution of vitamin C and PP was observed. It was noticed that the increase in gradient time (60 min) has only small effect on the last three peaks as a minor increase in the retention time in comparison with the steep gradient (20 min).

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Table 1. The retention time of the 12 basic runs and comparison of the retention time between the predicted and experimental runs at the optimum condition t_G20_*T*45°C

Vitamins	Basic runs' retention time, t_R (min)										Comparison between the predicted run and the experimental run for the standard mixture solution			
	Ternary eluent B: methanol 100%				Ternary eluent B: (methanol/acetonitrile) (50:50, v/v)				Ternary eluent B: acetonitrile 100%					
	t_G20_730°C	t_G20_760°C	t_G60_730°C	t_G60_760°C	t_G20_730°C	t_G20_760°C	t_G60_730°C	t_G60_760°C	t_G20_730°C	t_G20_760°C	t_G60_730°C	t_G60_760°C	Predicted retention time (min)	Experimental retention time (min)	Difference (min)	Accuracy (%)
B1	3.008	2.926	3.007	2.951	2.925	2.885	2.942	2.861	2.850	2.725	2.851	2.833	*3.390*	3.367	−0.023	99.32
Ascorbic acid	3.604	3.356	3.601	3.372	3.407	3.237	3.428	3.212	3.229*	3.067*	3.233*	3.221*	*4.400*	4.001	−0.399	90.93
PP	4.044	3.872	4.040	3.922	3.551	3.572	3.572	3.543					*5.334*	5.308	−0.026	99.51
B6	4.818	4.129	4.808	4.199	4.264	3.949	4.260	3.958	3.835	3.515	3.843	3.810	*5.920*	6.165	0.245	95.86
PABA	7.862	7.268	8.946	8.124	7.422	6.922	8.294	7.549	6.715	6.142	7.250	6.769	*8.583*	8.882	0.299	96.52
B12	10.486	9.814	18.153	16.324	8.950	8.485	14.512	13.408	7.601	7.325	11.301	10.558	*11.000*	11.050	−0.050	99.55
B2	11.774	10.971	20.001	17.874	9.850	9.318	15.518	14.283	8.253	7.925	12.025	11.238	*12.160*	12.249	0.089	99.27

*Ascorbic acid and nicotinamide (PP) coeluted.
The retention times of the vitamins at the optimum conditions at the virtual chromatograms are given in italics.

3.1.3. Runs where eluent B was acetonitrile

When the ternary eluent was composed of 100%, acetonitrile least retention was observed for all peaks compared to other organic eluents tested. Also, vitamin C and PP peaks coeluted in all the runs.

In the 12 basic runs, the effect of the three parameters (t_G, temperature, and ternary eluent) on the separation of the vitamins' mixture under investigation can be summarized as follows:

Linear gradient steepness had no significant effect on separation, especially for the first four peaks (B_1, C, PP, and B6), with only a minor prolongation in the retention time for the last three peaks (PABA, B_{12}, and B_2) when flatter gradients were used. On the other hand, when column temperature increased, retention times decreased, which was sometimes accompanied by improvement in resolution of peaks of vitamin C and PP when the ternary eluent was acetonitrile/methanol (50:50).

Increasing percentage of acetonitrile in eluent B led to decrease in the run time accompanied by worsening in resolution of the first four peaks.

Generally, behavior of the peaks followed a certain pattern in all runs due to the pH used for the mobile phase (pH 2.6): the first four peaks, showed a significantly similar retardation behavior at different experimental chromatographic conditions. The p*Ka* of these four peaks ranges from 4.2 to 5.0 that promotes their ionization and their conversion to a more polar form at pH 2.6. This later form is more water soluble, leading to less retardation. While for the last three peaks (p*Ka* range was1.59–2.50), they are unionized under the used pH that leads to more efficient retention on the stationary phase.

3.2 Peak tracking

Peak tracking refers to the matching of the peak of the same compound between runs where conditions have changed; it is an important step prior to DryLab® modeling. In the 3D modeling, the four experiments of the t_G-*T* model have to be aligned in a way, to show all data (t_R and peak area) of one peak in the same horizontal line for the three different eluent compositions. The peak identification procedure is carried out based on similarities in peak areas, which are fairly constant for the same compound in the four runs of t_G-*T* model. Peak areas represent sample mass (concentration × volume) and are constant in each individual run, fairly independent of other experimental conditions [17]. The chromatograms were exported to PeakMatch® software in AIA format (*.cdf), followed by a normalization with a factor of 1000, to ease handling of numbers representing peak areas. Tracking was completed successfully, and it showed that the elution order of the seven peaks did not change throughout runs.

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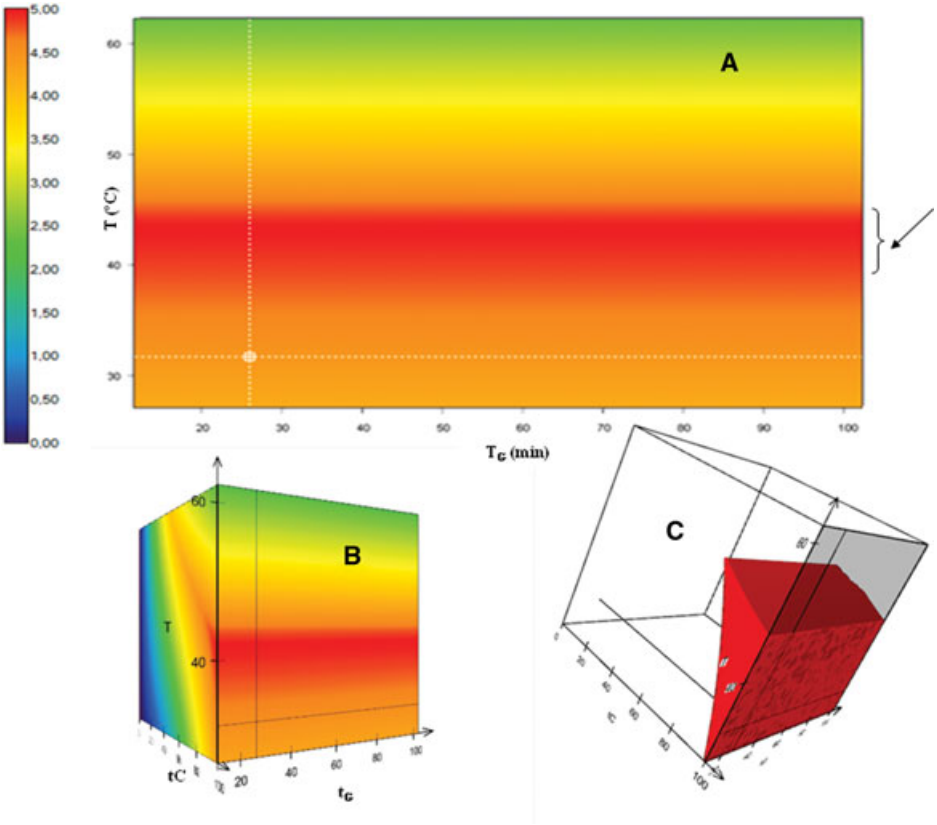


Figure 1. 3D resolution spaces where (A) showing the t_G-T(MeOH) plane; (B) 3D resolution spaces gradient time (t_G (min))–temperature (T (°C)–ternary eluent composition (t_C). Red color within the resolution spaces indicates operating region with $R_s > 1.5$, whereas blue color indicates operating region with $R_s = 0$ and (C) showing the design space (red color). The arrow in A points to the preliminary computed optimum conditions chosen for the following steps in the optimization.

3.3 Generation of the 3D resolution model (the cube)

Data obtained in PeakMatch were exported to DryLab®, which predicts the 3D resolution map, the cube, representing the simultaneous influence of three parameters (t_G, T, and t_C) on the chromatographic selectivity and critical resolution of the most closely eluting pair of peaks. The advantage of the 3D resolution volume is the fact, that the eight corner points and four intermediate points of the space are all measured and form a "cube" as a true "resolution space" as presented in Fig. 1, allowing for prediction of about 10^6 separations within the cube with an unparalleled precision of the retention times and chromatographic separation selectivity [16].

The critical resolution in a chromatogram is, by definition, the lowest resolution value between the least resolved peak pair, in our case among PP and B6. This pair is termed "the critical peak pair". The effect of the three previously mentioned parameters on the resolution of the critical peak pair can be viewed in different colors where the red color indicates the operating regions of maximum resolution where $R_s > 1.5$, and the blue color indicates the operating regions where $R_s = 0$ and in between these two colors a series of colors describe different degrees of resolution according to the legend as shown in Figs. 1 and 2.

The accuracy of DryLab® and the validity of the modeling process can be demonstrated by comparison of the predicted runs to the actual experimental runs as demonstrated in Fig. 3. The comparison proved that an excellent accuracy in predictions of retention times was obtained for peaks with an average of >97.3%.

The effect of the three parameters studied can be deducted from the 3D resolution map on the separation of mixture components, as follows:

Ternary eluent: Acetonitrile has been found to worsen R_s. In fact, when the percentage of acetonitrile increased in the eluent, the red region in the cube decreased in favor of the blue color which governed the map 40 to 100% acetonitrile as represented in Fig. 1. At these compositions of acetonitrile, no combination of the other operating parameters (t_G and T) could afford acceptable resolution. Hence, in separation of water-soluble vitamins, it will be a waste of time and money to use acetonitrile in eluent B. On the other hand, the use of 100% methanol as eluent B enhances dramatically the R_s of these vitamins.

Gradient time: It was found that variations in gradient time had no significant effect on the R_s of water-soluble vitamins. As a consequence, the optimum t_G is to be chosen to satisfy the least run time.

Temperature: It was found that increase in temperature leads to enhanced R_s of water-soluble vitamins till 45°C and followed by decrease in R_s at temperatures higher than this value.

To conclude the best resolution and least run time are obtained with the following conditions as demonstrated in Fig. 2; will be a linear gradient time of 20 min at a flow rate of

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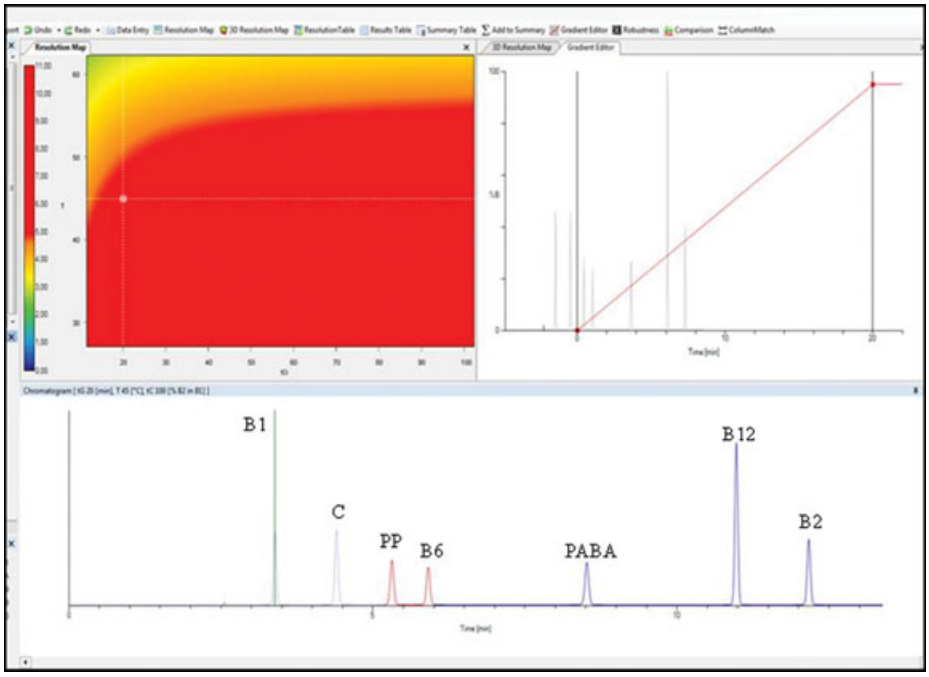


Figure 2. The resolution map showing t_G-T model at the optimum condition t_G20_T45°C, where eluent B is 100% methanol, at flow rate 1 mL/min.

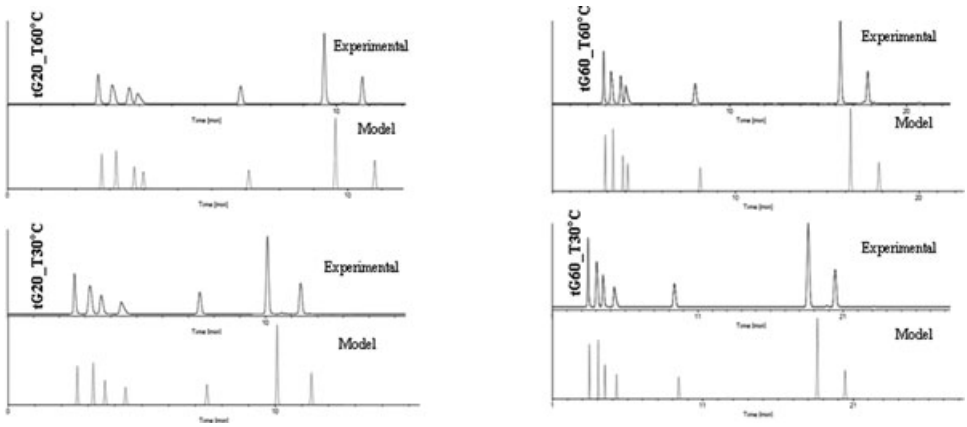


Figure 3. Comparison of the experimental and modeled chromatograms of the four basic runs, where eluent B is methanol 100%: t_G20_T30°C, t_G20_T60°C, t_G60_T30°C, t_G60_T60°C.

1 mL/min from 0 to 95% methanol as the organic modifier at pH 2.6 with the column kept at a temperature of 45°C. The critical peak pair was found to be PP (peak number 3) and vitamin B6 (peak number 4), as shown in Fig. 2. At the optimum computed conditions, the run time is only 13 min that will save time and money.

3.4 Implementation of the predicted optimum conditions and comparison between virtual and experimental runs for the laboratory prepared mixture

The HPLC system was conditioned at the previously mentioned optimum conditions of gradient design, temperature, and ternary eluent, and then the mixture was injected. The retention times of the seven peaks in the experimental chromatogram, as presented in Fig. 4, were 3.367 min (vitamin B1), 4.001 min (vitamin C), 5.308 min (PP), 6.165 min (vitamin B6), 8.882 min (PABA), 11.050 min (B12), and 12.249 min (B2), with an average percentage of accuracy 97.28%, as listed in Table 1. This method provided best separation in the least run time (13 min) with respect to the three influential parameters (gradient time, temperature, and ternary eluent composition). Also, the optimum ternary eluent composition was methanol 100%, which is favorable due to the shortage and the high price of the acetonitrile.

3.5 Implementation of the predicted run and comparison between virtual and experimental runs on the pharmaceutical preparations

3.5.1 Royal vit G capsules®

The "Royal vit G" soft gelatin capsule (SEDICO, Egypt) contains vitamin B1 (15 mg), PP (15 mg), B6 (1 mg), PABA

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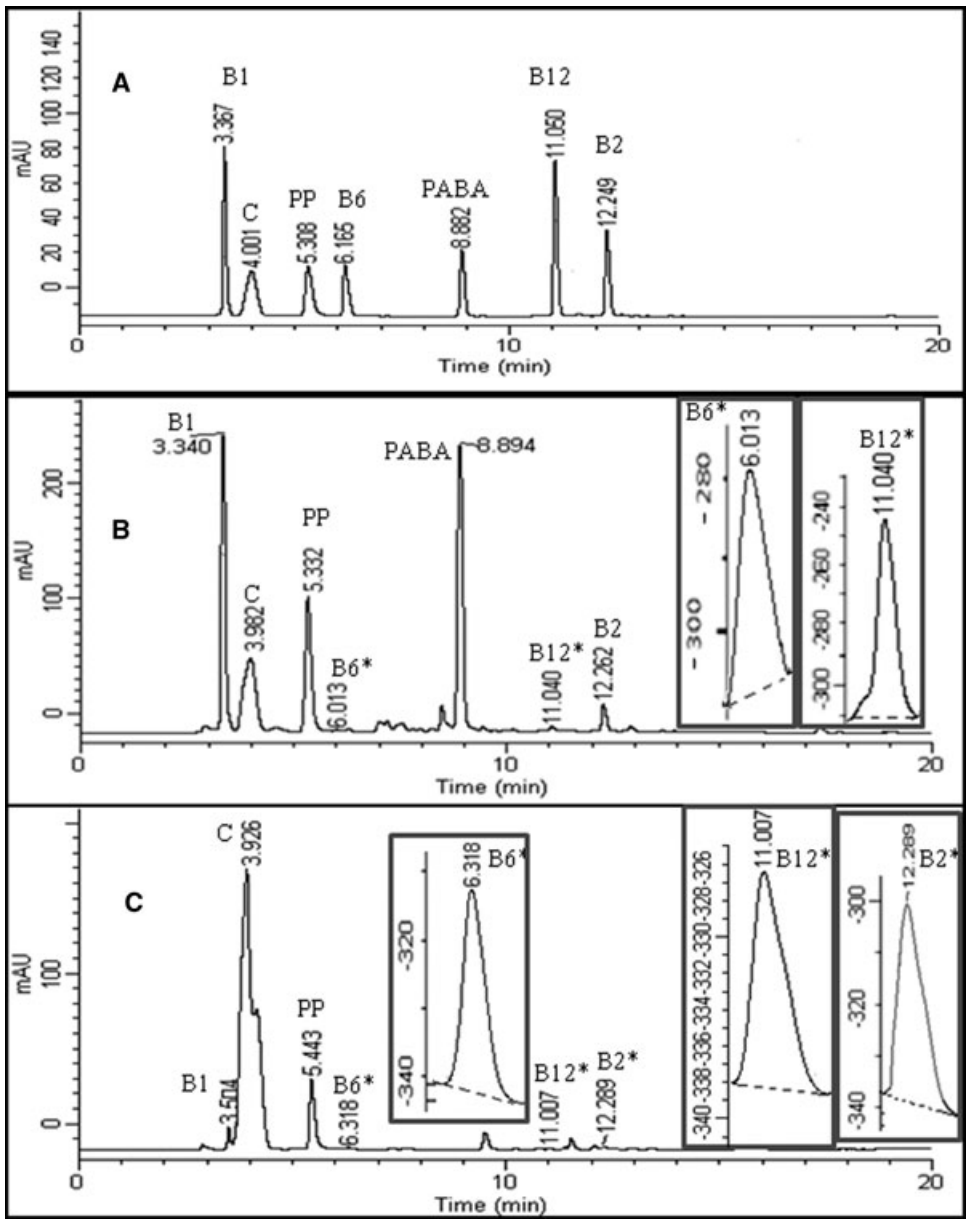


Figure 4. Chromatograms of the standard solution (A) and pharmaceutical preparations: Royal Vit G capsules®; (B) and sport drink Effervescent Instant®; (C) at the optimum condition named t_G20_745°C, where eluent B is methanol 100% at flow rate 1 mL/min. The insert box represents a zoom of the corresponding vitamins.

(50 mg), B12 (5 μg), and B2 (4 mg). The content of the capsule was dissolved in 25 mL 25 mM phosphate buffer pH 2.60 and filtered by membrane filter 0.45 μm. A total of 20 μL of the filtrate were injected at the optimum predicted conditions.

Retention times of the six peaks, presented in Fig. 4, were as follows: vitamin B1 3.340 min with a difference of 0.050 min than the virtual, for PP at 5.332 min with a difference of 0.002 min, vitamin B6 6.013 min with a difference of 0.093 min, PABA 8.894 min with a difference of 0.311 min, B12 11.040 min with a difference of 0.040 min, and B2 12.262 min with a difference of 0.102 min. The difference between the experimental and virtual retention times ranged from ±0.002 up to 0.3 min, which proved good correlation between the predicted model and the experimental model, with an average percentage of accuracy 97.66%.

A peak appeared at the same retention time of vitamin C (3.982 min), although the capsule is not labeled to contain vitamin C as one of the active ingredients. This can be explained by the presence of calcium ascorbate as an additive in the capsule. No significance change was noticed in the retention time difference between the standards and the capsule.

3.5.2 Sport drink Effervescent Instant®

The sport drink sachet (SEDICO, Egypt) contains vitamin B1 (1.30 mg), C (90 mg), PP (10 mg), B6 (1.70 mg), B12 (2.40 μg), and B2 (1.65 mg sachet). The content of the sachet was dissolved in 100 mL 25 mM phosphate buffer pH 2.60

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and filtered by membrane filter 0.45 μm. 20 μL of the filtrate were injected at the optimum predicted conditions.

At the experimental runs, retention time of the six peaks was as follow: vitamin B1 3.504 min with a difference of 0.114 min than the virtual, for C at 3.926 min with a difference of 0.474 min, for PP at 5.443 min with a difference of 0.109 min, vitamin B6 6.318 min with a difference of 0.398 min, B12 11.007 min with a difference of 0.007 min, and B2 12.289 min with a difference of 0.129 min. The difference between the experimental and virtual retention times ranged from ±0.007 up to 0.4 min, which proved good correlation between the predicted model and the experimental model, with an average percentage of accuracy 95.99%.

A shoulder was observed in the vitamin C peak due to column overload in view of the high concentration of this vitamin (9 mg/mL). It worth mentioning that this shoulder was not observed at all in the standard mixture solution. No significance difference existed in retention times between the laboratory prepared mixture and the sachet preparation. Only a minor difference of 0.2 min was observed for B1 and B6 peaks which may be due to difference in concentration of these two vitamins in the laboratory prepared mixture in comparison with the sachet B1: 0.1 mg/mL in the prepared mixture versus 0.01 mg/mL in the sachet, and B6: 1 mg/mL in the prepared mix versus 0.017 mg/mL in the sachet.

Although ultra performance liquid chromatography (UPLC) is considered a much faster technique of analysis and is being adopted in many factory sites, yet its cost and frequent troubleshooting are limiting its use in some third world country laboratories. Nevertheless, method transfer from HPLC to UPLC has proven to be simple and possible [24] and hence we would propose the same design space determined in this study can be extrapolated to UPLC analyses of water-soluble vitamins.

4 Concluding remarks

For the first time, the exact design space of RP-HPLC of seven water-soluble vitamins (B1, C, PP, B6, PABA, B12, and B2) commonly commercialized in pharmaceutical preparations has been accurately determined with respect to three critical operating parameters in HPLC separations gradient time, temperature, and ternary eluent mobile phase composition using the 3D modeling feature of DryLab® software. An optimized robust method is reported with respect to these three influential parameters to achieve best separation in the least analysis time (less than 20 min). The methodology presented in this work was found to be in correlation with QbD principles and results in the definition and visualization of the so-called design space. This will facilitate the interlaboratory method transfer and documentation. Also, due to the transferred from one ternary eluent to another, this method could be of a great help in case of its shortage, or its inflated price or in case of increasing the trends toward a greener environment.

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