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# Simultaneous microemulsion liquid chromatographic analysis of fat-soluble vitamins in pharmaceutical formulations: Optimization using genetic algorithm

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ABSTRACT

An environmentally benign and simple method has been proposed for separation and determination of fat-soluble vitamins using isocratic microemulsion liquid chromatography. Optimization of parameters affecting the separation selectivity and efficiency including surfactant concentration, percent of cosurfactant (1-butanol), and percent of organic oily solvent (diethyl ether), temperature and pH were performed simultaneously using genetic algorithm method. A new software package, MLR-GA, was developed for this purpose. The results indicated that 73.6 mM sodium dodecyl sulfate, 13.64% (v/v) 1-butanol, 0.48% (v/v) diethyl ether, column temperature of 32.5 °C and 0.02 M phosphate buffer of pH 6.99 are the best conditions for separation of fat-soluble vitamins. At the optimized conditions, the calibration plots for the vitamins were obtained and detection limits (1.06–3.69 μg mL$^{-1}$), accuracy (recoveries > 94.3), precision (RSD < 3.96) and linearity (0.01–10 mg mL$^{-1}$) were estimated. Finally, the amount of vitamins in multivitamin syrup and a sample of fish oil capsule were determined. The results showed a good agreement with those reported by manufactures.



## 1. Introduction

Vitamins are a group of organic compounds that are, in very small amounts, essential for normal metabolism, growth and development, and regulation of cell functions. Thirteen vitamins are recognized in human nutrition which may be conveniently classified into two groups according to their solubility: as water-soluble (B-complex and C) and fat-soluble (A, D, E and K) [1].

Fat-soluble vitamins have been analyzed in a variety of sample matrices [2,3]. Up to the present, the method of choice for determining the fat-soluble vitamins has been high-performance liquid chromatography (HPLC) and several HPLC methodologies have been reported for their determination [4–14]. In most of these methods, large amounts of organic solvents are used as the mobile phase, which result in an environmental impact. Application of micellar media has been proposed to satisfy the society need for the development of green analytical methods [15–17]. But micellar eluent suffer from poor separation efficiency. Microemulsions are the key to this problem, since they offer both the lower toxicity of the mobile phase and high separation efficiency. Microemulsions are dispersions of nanometer-sized droplets of an immiscible liquid within another liquid which is facilitated by the addition of surfactants and often also cosurfactants. Microemulsions are classified as either oil-in-water (O/W) or water-in-oil (W/O) [18]. There has been a recent increase in the use of microemulsion eluents in HPLC which has been referred as microemulsion liquid chromatography (MELC) [19–24].

Using a microemulsion as the mobile phase alters the solutes partitioning between the mobile and stationary phase characteristics in comparison with conventional HPLC because a layer of surfactant molecules adsorbs on the surface of the stationary phase. The solutes also partition from the aqueous phase or the stationary phase onto the microemulsion droplets. The eluent parameters that mainly affect separations in MELC are surfactant concentration, percent of a small alcohol as a cosurfactant, and percent of organic oily solvent, temperature and pH of the mobile phase [18]. When there are many parameters and especially if the factors interact, application of experimental designs has been proposed to optimized separation condition and reduces the number of experiments and amounts of chemicals. MELC separations are affected by more than two factors as compared with hydro-organic reversed-phase HPLC. For this reason, it is necessary to simultaneously optimize the factors affecting the separation. This approach was used by Jancic et al. [21] in the analysis of fosinopril sodium and fosinoprilat separation applying the MELC. But

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they did not optimize all the factors that mainly affect separation simultaneously.

Application of optimization algorithms such as the genetic algorithm (GA) after modeling the system by multiple linear regression (MLR) algorithms result in the desired optimized experimental conditions with faster convergence [25,26]. GA emulates the biological evolutionary theory and allows finding a global, true optimized condition among several possible local alternatives. GA optimization has been used in many areas, and it has been applied in chromatographic optimization as well [27,28].

In this work, a method for the determination of five fat-soluble vitamins in pharmaceutical formulations was developed and validated using an O/W microemulsion as HPLC eluent. For the first time effect of all the eluent parameters that mainly affect separations in MELC, were optimized simultaneously. A new user-friendly software (MLR-GA) was developed to apply GA to these models and find the operational conditions that provide the minimized chromatographic responses for all evaluated analytes simultaneously. The modified chromatographic exponential function (MCEF) [27], an objective function in terms of resolution and analysis time, was minimized through the GA algorithm.

## 2. Experimental

### 2.1. Reagents and solutions

Analytical-grade cholecalciferol (Vitamin $D_3$), retinol palmitate (Vitamin A), phylloquinone (Vitamin $K_1$) and α-tocopherol acetate (Vitamin E) were supplied by Roche (Basel, Switzerland). HPLC-grade methanol, analytical-grade α-tocopherol and reagent-grade ethanol, 1-butanol (BuOH), diethyl ether (DEE), cyclohexane, cyclohexanol, n-heptanol, n-hexane, dimethyl solfoxide (DMSO), sodium dodecyl sulfate (SDS), sodium dihydrogen phosphate, sodium monohydrogen phosphate, phosphoric acid and sodium hydroxide all were purchased from Merck (Darmstadt, Germany). Doubly distilled, deionized water was used in all experiments. Stock standard solution of each vitamin and α-tocopherol were prepared in 1-butanol containing 1% of ascorbic acid to provide a concentration of 30 mg $mL^{-1}$ for all the compounds. These solutions were stable for at least 1 month when stored below 4 °C and protected from light. Working solutions were prepared just before performing the analysis from the stock solutions by appropriate dilution with ethanol to the desired concentration levels. The components of the microemulsion were appropriate volumes of 0.5 M SDS, 1-butanol, diethyl ether and phosphate buffer (0.02 M). These components were mixed together and the pH was adjusted to the desired value using phosphoric acid or sodium hydroxide. Then the mixture was treated on an ultrasonic bath (Tecno-GAZ, Italy) for 30 min. The resulting transparent mobile phase and all other solutions were filtered using 0.45 μm membranes (Millipore, Bedford, MA, USA).

### 2.2. Apparatus

The chromatographic measurements were carried out with an Agilent Technologies (Wilmington, DE, USA) 1100 HPLC system equipped with Standard Micro Auto Sampler Model G1313A, Micro Vacuum Degasser Model G1379A, Quaternary Pump Model G1311A, Series Multiple Wavelength Detector Model G13658 and a Zorbax-eclipse XDB-$C_8$ (150 mm × 4.6 mm, 5 μm) column. The analytical column was water-jacketed and thermostated with a Lauda Ecoline Staredition RE104 Water Bath (Germany). The chromatographic calculations were performed using a Chemstation data handling system. Before analysis, the column was conditioned by flowing the mobile phase through the system for 45 min at 0.9 mL/min. During the separation, the mobile phase flow rate was kept at 0.9 mL/min (due to high back pressure of microemulsions) and peaks were detected at 285 nm.

**Table 1**
Effect of oily organic solvent on separation of vitamins.

| Oil | $R_{S1}$ | $R_{S2}$ | $R_{S3}$ | $R_{S4}$ | $t_f$ | MCEF |
|---|---|---|---|---|---|---|
| None | 2.50 | 1.31 | 0.59 | 5.99 | 113.02 | 37.16 |
| Cyclohexane | 4.50 | 1.58 | 0.00 | 5.70 | 42.60 | 48.64 |
| Cyclohexanol | 3.25 | 1.14 | 0.55 | 4.97 | 80.17 | 32.15 |
| Heptanol | 2.10 | 0.82 | 0.50 | 3.82 | 21.36 | 20.28 |
| Diethyl ether | 5.15 | 2.36 | 0.65 | 8.70 | 47.66 | 15.71 |

Conditions: column, Zorbax-eclipse XDB-$C_8$ (150 mm × 4.6 mm, 5 μm), mobile phase, 50.0 mM SDS, 10.0% 1-butanol (v/v), 1.0% oily organic solvent (v/v), 20.0 mM phosphate buffer pH 6.73; 5 μL sample; flow rate 0.9 mL/min; column temperature 35.0 °C; λ, 285 nm.

### 2.3. Sample preparation

Five milliliters of multivitamin syrup (Amin Pharmaceutical, Isfahan, Iran) or the total content of a pure cod liver oil capsule (Seven seas, England) was transferred to a test tube, and 0.25 g of ascorbic acid, 3 mL of n-hexane–DEE solution (9:1 (v/v)) and 2 mL DMSO were added. The sample was vortexed continuously for 5 min and then centrifuged for 5 min at 3000 rpm. The upper organic layer was transferred to a 25 mL volumetric flask. This procedure was repeated four more times. The organic phases were joined, evaporated to dryness at 30 °C and the residue was re-dissolved in ethanol [29]. After the extraction processes, the samples were filtered through a 0.45 μm membrane filter and injected into the chromatographic system for analysis.

## 3. Results and discussion

### 3.1. Effect of organic oily solvent

Various mobile phases have been studied in order to optimize the separation of fat-soluble vitamins using reversed-phase HPLC. Since these compounds are insoluble in water, the major constituent in the mobile phase should be an organic solvent, e.g. methanol or acetonitrile. In order to achieve the desired retention and to increase selectivity, water or stronger organic modifiers have been used in few percents.

We have previously reported the separation of Vitamins E and A using micellar mobile phase and a 10 cm $C_{18}$ column [15], in which the mobile phase major constituent was water (>85%). But our effort for MLC separation of all fat-soluble vitamins i.e. E, $D_3$, $K_1$, A and α-tocopherol was failed even with using a longer column and long analysis time. Because of the oily nature of fat-soluble vitamins which are strongly adsorbed on a reversed-phase stationary phase with high carbon loading, here we try to separate them using isocratic MLC on a $C_8$ stationary phase. However, as can be seen in Table 1, poor resolution was obtained in more than 100 min. The ability of O/W microemulsions to solubilise highly hydrophobic sample matrices has been approved [20]. To improve the separation, introduction of a second equilibrium, i.e., partition from the aqueous phase or the stationary phase onto the microemulsion droplets, was investigated by addition of the organic oily solvent (<1%). Different compounds including DEE, cyclohexane, cyclohexanol and n-heptanol were examined. The results (Table 1) show that usually a considerable improvement in separation was obtained by addition of such a solvent, while the mobile phase major constituent is still water (>85%). Using MECF as the criterion (Eq. (1)), the best results were obtained using DEE as the organic oily solvent. Addition of DEE, decreases retention of the vitamins because of the higher distribution of the solutes to the microemulsion droplets and the selectivity of the separation was significantly affected.