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Comparing monolithic and fused core HPLC columns for fast chromatographic analysis of fat-soluble vitamins

SAID EL KURDI[1]
DINA ABU MUAILEQ[2]
HASSAN A. ALHAZMI[3]
MOHAMMED AL BRATTY[3]
SAMI EL DEEB[4]*

[1] *Chemistry Department Faculty of Science, Islamic University Gaza, Palestine*

[2] *Pharmaceutical Chemistry Department, Faculty of Pharmacy Al-Azhar University-Gaza, Gaza Palestine*

[3] *Pharmaceutical Chemistry Department, Faculty of Pharmacy Jazan University, Jazan, Saudi Arabia*

[4] *Institute of Medicinal and Pharmaceutical Chemistry TU Braunschweig, D-38106 Braunschweig, Germany*

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HPLC stationary phases of monolithic and fused core type can be used to achieve fast chromatographic separation as an alternative to UPLC. In this study, monolithic and fused core stationary phases are compared for fast separation of four fat-soluble vitamins. Three new methods on the first and second generation monolithic silica RP-18e columns and a fused core pentafluoro-phenyl propyl column were developed. Application of three fused core columns offered comparable separations of retinyl palmitate, *DL*-α-tocopheryl acetate, cholecalciferol and menadione in terms of elution speed and separation efficiency. Separation was achieved in approx. 5 min with good resolution ($R_s > 5$) and precision (RSD ≤ 0.6 %). Monolithic columns showed, however, a higher number of theoretical plates, better precision and lower column backpressure than the fused core column. The three developed methods were successfully applied to separate and quantitate fat-soluble vitamins in commercial products.

Keywords: monolithic silica column, fused core column, HPLC, fat-soluble vitamins, analytical performance

There is an increasing demand for fast chromatographic methods suitable for routine analyses with low analysis cost. So far, HPLC is the main instrumental technique used widely for the separation and quantification of drugs. Accordingly, there is an enormous need for highly efficient, selective LC columns to provide faster and more precise analyses, especially for high throughput samples in research laboratories and pharmaceutical industry. Three approaches are currently in competition to achieve fast chromatographic analysis UPLC (ultra performance liquid chromatography), fused core columns and monolithic columns, attached to HPLC (1, 2).

UPLC relies on the use of short columns with sub-2-μm particles. The UPLC instrument is designed to resist pressure up to 1.5×10^8 Pa, which exceeds the pressure limits for

* Correspondence; e-mail: s.eldeeb@tu-bs.de

conventional HPLC instrumentation. The small particle size stationary phase offers a large surface area, resulting in increased separation efficiency, which allows for the use of a shorter column, thus achieving faster analysis (3). However, the application of this approach requires replacing the conventional HPLC instrument with a new UPLC instrument, which is costly and hence unfavorable to the users.

Another option for fast chromatographic analysis is the use of fused core columns containing particles with a solid non-porous core of 1.7 μm coated with a porous layer of 0.5 μm with a shell volume typically 60–75 % of the particle volume and with a much narrower particle size distribution than that of standard, fully porous particles (4). Such particles, with 9-nm pore size in the porous shell, provide approximately 150 $m^2 g^{-1}$ specific surface area (5). They exhibit high efficiency and a short diffusion path of the analyte and faster mass transfer from the mobile phase into and out of the porous layer of the particles than non-porous particles (6). The fused core pentafluoro-phenyl propyl (PFP) stationary phase (Ascentis® Express F5 column) provides a reversed-phase packing with electron-deficient phenyl rings because of electronegative fluorines. This phase retains compounds by forming π-π interactions, possible steric interactions with the bonded phase, in addition to polar interactions (6).

A third approach for fast HPLC analysis is the use of monolithic silica stationary phases. The first generation reversed phase monolith (Chromolith Performance® RP 18e) is composed of a continuous piece of porous silica, produced using a sol-gel process leading to rod columns with a bimodal pore structure. The bimodal pore structure shows a special combination of macropores and mesopores; mesopores form a fine porous structure (average pore size 13 nm) and create a large uniform specific surface area (300 $m^2 g^{-1}$) on which adsorption occurs to enable separation. Macropores (average size 2 μm) allow rapid flow of the mobile phase at low pressure. High permeability of the column allows for the use of a high flow rate without development of high backpressure and thus enables fast analysis (1, 7).

The first generation monolithic silica RP-18e columns have been successfully applied in method transfer from conventional particle packed columns or for the development of new fast methods (8–17). Furthermore, application of the flow programming elution mode with monolithic silica RP-18e columns produced ultra-fast methods of just a few seconds with good precision (14, 18, 19). In 2011, the second generation monolith (Chromolith HighResolution® RP 18e) became commercially available. This new monolithic column differs from the first generation monolith in pore size, with smaller macropores (1.15 μm) to improve the peak shape (20, 21), and larger mesopores of 15 nm that provide 250 $m^2 g^{-1}$ specific surface area to enable even better separation. This type of monolith is characterized by a much more homogeneous porous silica structure than that of the first generation. Some successful applications of the second generation monolith silica RP-18e columns have been already published (22, 23).

Four fat-soluble vitamins (FSVs), namely, menadione (vitamin K3), cholecalciferol (vitamin D3), retinyl palmitate (vitamin A) and *DL*-α-tocopheryl acetate (vitamin E) were selected as a model for the comparative study between the PFP fused core and the two, first and second, monolith silica RP-18e column technologies, regarding separation efficiency, precision and analysis time. These vitamins represent the actual mixture found in a number of different dosage forms, some considered as drugs and others as dietary supplements (24, 25).

This work aims to achieve fast and appropriate methods suitable for large scale analysis to save cost and time.

EXPERIMENTAL

Materials

Acetonitrile and methanol, both HPLC grade, were obtained from LAB-Scan (Poland). Distilled water was produced by GFL (Germany). Menadione standard was obtained from Sigma Aldrich (China), cholecalciferol from Sigma Aldrich (Poland), retinyl palmitate and *DL*-α-tocopheryl acetate are both from Sigma Aldrich (Switzerland). Vitamin E capsules and pediatric drops containing both vitamin D and A, used in this study as application products, were purchased locally.

Instruments and software

LC analysis was carried out using an Agilent 1260 (Agilent Technologies, Germany) equipped with an Agilent 1260 Infinity Quaternary Pump with vacuum degasser (G1311B) and diode array detector (G4212B). The software was Agilent ChemStation B.04.02.

Ascentis® Express F5 (2.7 μm, 10 cm × 4.6 mm) column was purchased from SUPELCO, USA, Chromolith® Performance RP-18e (10 cm × 3 mm) and Chromolith® HighResolution RP-18e (10 cm × 4.6 mm) were from Merck KGaA, Germany.

Table I. Comparison between the methods developed on three columns

Parameter	Ascentis® Express F5 column				Chromolith® Performance RP-18e				Chromolith® HighResolution RP-18e			
Mobile phase	ACN/MeOH/H_2O (10:80:10)				ACN/MeOH (35:65)				ACN/MeOH (25:75)			
Flow rate (mL min^{-1})	1.8				1.5				4			
Injection volume (μL)	50				50				50			
R_s (critical peak pair)	(K3, D3) = 3.82 (E, A) = 3.1				(D3, E) = 3.176				(D3, E) = 3.37			
t_R (min)	K3	D3	E	A	K3	D3	E	A	K3	D3	E	A
	0.72	1.13	2.76	3.51	0.44	1.26	1.58	4.42	0.40	0.99	1.27	3.05
N^a	K3	D3	E	A	K3	D3	E	A	K3	D3	E	A
	908.19	1130.17	2700.9	2434.6	649.4	2451.45	3248.13	4263.19	650.23	3365.78	4547.44	6874.4
Run time (min)	4				5				4			
Column backpressure (Pa)	255 x 10^5				34.5 x 10^5				110 x 10^5			
Elution order	K3, D3, E, A				K3, D3, E, A				K3, D3, E, A			
Symmetry factor	K3	D3	E	A	K3	D3	E	A	K3	D3	E	A
	1.34	1.37	1.28	1.63	0.91	1.31	1.28	1.5	0.77	0.98	0.94	0.819

[a] Number of theoretical plates per 100 mm column length.

Chromatographic conditions

Chromatographic parameters for FSV separation on three columns are summarized in Table I. Wavelength programming was applied in the methods with λ_{max} of 250 nm for vitamin K3, 254 nm for vitamin D3, 284 nm for vitamin E and 325 nm for vitamin A.

Sample preparation and linearity level

FSV standards were prepared in ACN/MeOH (60:40) diluent in the following concentrations: vitamin K3 (0.04 mg mL^{-1}), vitamin D3 (0.1 mg mL^{-1}), vitamin E (1 mg mL^{-1}) and vitamin A (0.5 mg mL^{-1}). Sonication was used when required. FSV stock solutions were wrapped in aluminum foil and stored in refrigerator at 4.0 °C.

Aliquots of 1.5 mL of vitamin D3 and vitamin K3 and 3.0 mL of vitamin E and 1.0 mL of vitamin A standard solution were mixed and diluted up to 50.0 mL with the diluent to prepare a mixed standard solution. Linearity levels were achieved by subsequent dilution for each standard using the same diluent to get 5 levels for each vitamin.

Method validation

For each vitamin, five concentration levels were injected into 3 replicates on the three columns using the peak area response and concentration values to set calibration curves. Injected volume was 50 μL.

The analyte concentration that provided a signal to noise ratio (S/N) of < 3 was considered as *LOD* and the analyte concentration with (S/N) < 10 was considered as *LOQ* (26).

Method precision on each column was tested. RSD values for retention time (t_R) and peak area of vitamin K3 (4 μg mL^{-1}), D3 (4 μg mL^{-1}), E (40 μg mL^{-1}) and A (4 μg mL^{-1}) on each of the three columns were calculated.

Preparation of dosage forms for analysis

Two different types of vitamin-containing products, vitamin E capsules and pediatric drops containing both vitamin D and A were used for the application of the three newly developed methods. Vitamin E capsule containing 400 mg *DL*-α-tocopheryl acetate was extracted with 50 mL ethanol (HPLC grade, ≥ 99.8 %) by sonication for 5 min, followed by centrifugation and 25x dilution with ACN/MeOH (60:40) to get a concentration of 320 μg mL^{-1}.

The tested pediatric drops container was labeled to contain cholecalciferol (0.1 mg mL^{-1}) and retinyl palmitate (5.5 mg mL^{-1}). Samples of these pediatric drops were prepared by diluting 1 mL with ACN/MeOH (60:40) diluent and spiking with 2 mL of 1 mg mL^{-1} vitamin D3 standard solution in a 50-mL volumetric flask to get a concentration of 110 μg mL^{-1} of retinyl palmitate and 42 μg mL^{-1} of cholecalciferol.

RESULTS AND DISCUSSION

Good separation of the four FSVs was achieved in less than 5 min with each of the three tested columns. The three columns gave the same elution order for the four separated FSVs. The chromatographic elution patterns on each of the three columns are shown in Fig. 1.

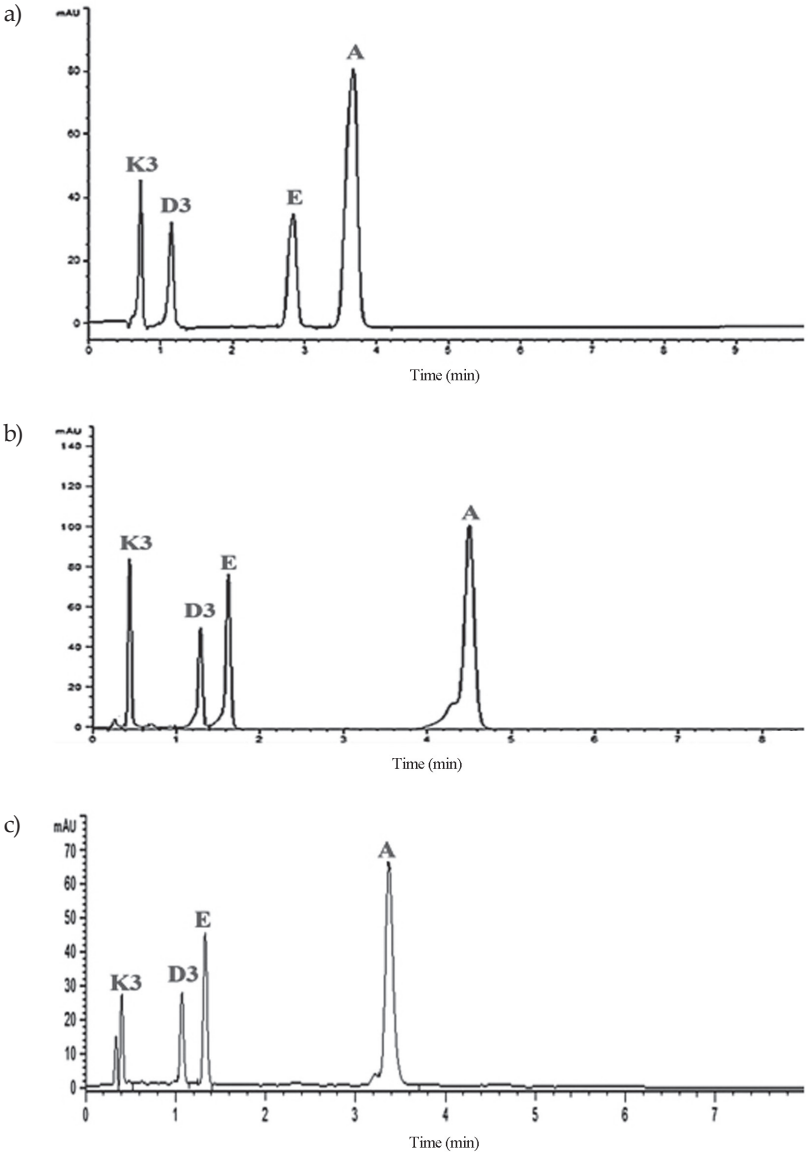

Fig. 1. Chromatographic separation of the four FSVs on: a) Ascentis® Express F5 column (2.7 μm, 10 cm × 4.6 mm), mobile phase (ACN/MeOH: H_2O, 10:80:10), flow rate: 1.8 mL min^{-1}, wavelength programming (250 nm: 0–0.9 min, 254 nm: 0.9–2 min, 284 nm: 2–3.2 min, 325 nm: 3.2–5 min), b) Chromolith® Performance RP-18e column (10 cm × 3mm), mobile phase (ACN/MeOH, 35:65), flow rate: 1.5 mL min^{-1}, wavelength programming (250 nm: 0–1 min, 254 nm: 1–1.45 min, 284 nm: 1.45–3 min, 325 nm: 3–5 min), c) Chromolith® HighResolution RP-18e (10 cm × 4.6 mm), mobile phase (ACN/MeOH, 25:75), flow rate: 4 mL min^{-1}, wavelength programming (250 nm: 0–0.8 min, 254 nm: 0.8–1.2 min, 284 nm: 1.2–2 min, 3215 nm: 2–4 min).

Method validation

The methods were shown to be selective for the targeted analytes without peak interferences, where peaks' identity and purity were assured using UV-scan overlay of the diode-array detector. Resolution values (R_s) between critical peak pairs in the three developed methods exceed 3. All numerical values that compare between the three methods are also listed in Table I.

Regarding robustness, minor changes in organic solvent concentrations were examined during method development, in which separation efficiency was not affected. In this

Table II. LOD, LOQ, and linearity range for FSVs on Ascentis® Express F5, Chromolith® Performance RP-18e and Chromolith® HighResolution RP-18e columns

Linearity equation	Concentration levels[a]	R^2	Linearity range (μg mL^{-1})	LOQ	LOD	Column	Vitamin
$Y = 0.2109g + 14.105$	5	1.0000	0.068–40	68.0 ng mL^{-1}	20.0 ng mL^{-1}	Ascentis® Express F5 column	K3
$Y = 0.0815g + 72.499$	5	0.9987	0.02–100	20.0 ng mL^{-1}	6.0 ng mL^{-1}		D3
$Y = 0.0075g - 46.383$	5	0.9994	11–1000	11.0 μg mL^{-1}	3.4 μg mL^{-1}		Tocopheryl acetate
$Y = 0.132g + 32.427$	5	0.9999	0.01–150	10.0 ng mL^{-1}	3.0 ng mL^{-1}		Retinyl palmitate
$Y = 0.2465g + 127.42$	5	0.9994	0.048–40	48.0 ng mL^{-1}	14.4 ng mL^{-1}	Chromolith® Performance RP-18e column	K3
$Y = 0.093g - 5.3438$	5	0.9998	0.018–100	18.0 ng mL^{-1}	5.4 ng mL^{-1}		D3
$Y = 0.0082g + 1.8091$	5	0.9987	9.37–1000	9.4 μg mL^{-1}	2.8 μg mL^{-1}		Tocopheryl acetate
$Y = 0.1729g + 11.39$	5	0.9999	0.009–150	9.0 ng mL^{-1}	2.7 ng mL^{-1}		Retinyl palmitate
$Y = 0.0874g + 25.077$	5	0.9997	0.094–40	94.0 ng mL^{-1}	28.0 ng mL^{-1}	Chromolith® HighResolution RP-18e column	K3
$Y = 0.0335g + 23.479$	5	0.9996	0.02–100	20.0 ng mL^{-1}	6.0 ng mL^{-1}		D3
$Y = 0.0032g + 13.763$	5	0.9999	11–1000	11.0 μg mL^{-1}	3.3 μg mL^{-1}		Tocopheryl acetate
$Y = 0.0627g + 0.9085$	5	1.0000	0.011–150	11.3 ng mL^{-1}	3.4 ng mL^{-1}		Retinyl palmitate

[a] $n = 3$

study, minor changes in room temperature did not show significant changes in resolution or method selectivity.

Linearity, LOD *and* LOQ. – Calibration curves for each vitamin on the three columns were constructed with the regression coefficient and linearity equations over the defined linearity ranges (Table II). The obtained *LOD* and *LOQ* for each vitamin on the three columns are also given in Table II.

It was noticed that FSVs showed higher sensitivity in Chromolith® Performance RP-18. This can be attributed to its lowest operating flow rate and its smaller diameter (3 mm) compared to other columns (4.6 mm). When the column internal diameter is decreased, an increase in sensitivity (2–3 folds) can be expected when injecting the same analyte mass. This is due to increased analyte concentration in the mobile phase (27). The other columns showed comparable values; Chromolith® HR RP-18 and Ascentis® Express F5 still showed good sensitivity and acceptable *LOD* and *LOQ*.

Precision. – Graphical presentation of t_R and peak area RSD values is given in Fig. 2. All three columns in this study have shown good repeatability with RSD below 1 % and intermediate precision RSD below 2 %.

It is worth noting that Chromolith columns are easier to clean between consecutive runs than fused core columns and this may be a reason for better precision.

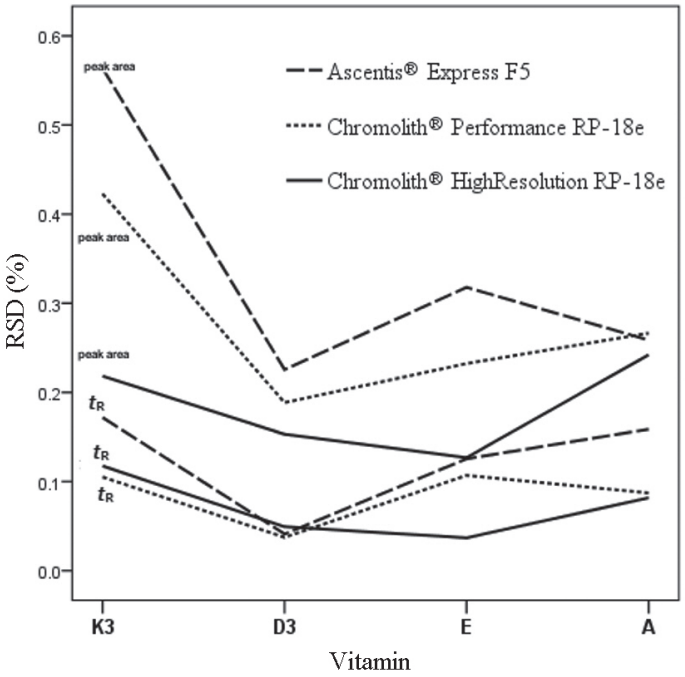

Fig. 2. RSD of retention time and peak area for FSVs on Ascentis® Express F5 Chromolith® Performance RP-18e and Chromolith® HighResolution RP-18e columns.

Monolithic versus fused core columns

Table I compares three columns in terms of separation efficiency, system backpressure, analysis time and peak symmetry. Monolithic silica RP-18e columns produced much lower system backpressure compared to the fused core column even at higher flow rates, also considering different compositions and viscosities of the mobile phases used in each column. The second generation monolithic RP-18e gave sharper peaks, as seen in Fig. 1c, and slightly better precision values (Fig. 2). On the other hand, the PFP fused core column showed the advantage of consuming less organic mobile phase. The PFP fused core column is claimed to be able to act as a reverse, normal and hydrophilic interaction liquid chromatography (HILIC) phase by controlling the mobile phase composition.

An increase in ACN proportion in the mobile phase was found to decrease retention of FSVs on the PFP fused core column, probably due to competitive π-π interaction of ACN with the solutes and stationary phase (28). The optimal ACN content in the mobile phase was found to be 10 %. This probably indicates that multiple mechanisms affect the separation in this column as both reversed phase and PFP chemistry contribute.

Peak tailing is often measured by the peak asymmetry factor (As); most column manufacturers consider asymmetry factors of 0.9-1.2 acceptable for test compounds. PFP fused core and first generation monolithic columns show slight peak tailing while the second generation monolithic column shows better symmetry values with sharper peaks. However, the Center for Drug Evaluation and Research (CDER) states that the tailing factor equal to or less than 2 is acceptable (29).

In method development on the PFP fused core column, it was first noted that the four FSVs primarily co-eluted in less than 3 minutes when using a pure ACN mobile phase. Good separation was obtained using 10 % ACN and 80 % MeOH in the mobile phase, since methanol participates in H-bonding interaction with the stationary phase and analytes. Water (10 %) was used to slow down elution strength in order to obtain satisfactory resolution between the eluted vitamins (separation was not possible without water in the mobile phase). The mobile phase looks to be HILIC, since it consists of water/organic phase 10:90 with just 10 % ACN. However, the separation of polar compounds on the HILIC stationary phase should, in principle, have an inverse order of elution than that observed by the reversed phase (29), which rejects the idea that the separation mechanism in this method depends only on simple partitioning of analytes on the adsorbed water layer. Instead, the analytes are probably retained in the pentaflouro-phenyl stationary phase by H-bonding, π-π interaction, dipole-dipole and charge transfer interaction. It has been noted that retention time increases with the increase of hydrophobic (non-polar) surface area of a compound. Branched chain compounds elute more rapidly than their corresponding linear isomers because of the overall surface area decrease (31). Accordingly, the mostly polar analyte, menadione (K3), eluted first, followed by cholecalciferol (D3), which has a hydroxyl group and branching structure, then tocopheryl acetate (E), which has an ester and ether functional groups and, lastly, retinyl palmitate (A), which has an ester group attached to a long aliphatic side-chain of palmitic acid. This order of elution coincides with the ascending order of the molecular mass and partition coefficient of the four tested FSVs.

Method development on RP monolithic columns was more straightforward than that on PFP fused core columns. The mechanism of separation relies mainly on hydrophobic interactions of analytes with alkyl chains in the stationary phase. Binding of the analyte

Table III. Dosage form analyses on Ascentis® Express F5, Chromolith® Performance RP-18e and Chromolith® HighResolution RP-18e

Dosage form	Vitamin	Column	Label mass	Mass found
Capsule	*DL*-α-tocopheryl acetate	Ascentis® Express F5	400 mg	345.36 mg
		Chromolith® Performance RP-18e		344.83 mg
		Chromolith® HighResolution RP-18e		344.12 mg
Pediatric drops	Cholecalciferol	Ascentis® Express F5	2100 μg	2089.15 μg
		Chromolith® Performance RP-18e		2089.45 μg
		Chromolith® HighResolution RP-18e		2089.05 μg
	Retinyl palmitate	Ascentis® Express F5	5500 μg	5465.00 μg
		Chromolith® Performance RP-18e		5463.00 μg
		Chromolith® HighResolution RP-18e		5464.50 μg

to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand on the stationary phase. In this study, a binary mobile phase composed of ACN and MeOH has been used, in which the best proportion of ACN was 35 % on the first generation monolithic RP-18e column and 25 % on the second generation monolithic RP-18e column. The elution order obeys partition coefficient values.

Analytical application

It is well known that the quality of multivitamin products should be controlled to avoid adulteration and to ensure that vitamin concentration does not reach toxic levels, especially for FSVs. As shown in Table III, the same results for each dosage form were obtained by each developed method. Vitamin E capsule assay results were found to be about 86 % while pediatric drops assay showed about 99 % of cholecalciferol and retinyl palmitate, complying with the uniformity of content in dietary supplement oral dosage form requirements (32).

CONCLUSIONS

Three LC columns, Ascentis® Express F5, Chromolith® Performance RP-18e and Chromolith® HighResolution RP-18e, were applied to compare the fused core and monolithic technologies when applied to the assay of four fat-soluble vitamins mixture.

In this study, three columns were compared with regard to separation efficiency, precision, linearity range, analysis time, system backpressure and elution order. Three simple isocratic HPLC methods were developed on these columns differing in mobile phase composition to provide good, efficient and fast analysis of the four FSVs. The overall analysis

time did not exceed 5 min with RSD values below 0.6 % for both retention time and peak area of each vitamin on the three columns. The linear concentration range for the three methods can accommodate various concentrations in dosage forms.

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