DEVELOPMENT AND VALIDATION OF PCR, PLS, AND TLC DENSITOMETRIC METHODS FOR THE SIMULTANEOUS DETERMINATION OF VITAMINS B_1, B_6 AND B_{12} IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

This work represents the simultaneous determination of thiamine hydrochloride (B_1), pyridoxine hydrochloride (B_6) and cyanocobalamine (B_{12}) by two different methods namely spectrophotometry multivariate calibration and densitometry. The spectrophotometric numerical method depends on the use of spectrophotometric data coupled to PLS and PCR multivariate calibration methods for the simultaneous determination of (B_1) and (B_6) in the presence of (B_{12}) in laboratory prepared mixtures and commercial tablets. A calibration set was prepared, where the three vitamins were modeled using a full factorial 2^3 with three center points experimental design. This calibration set was used to build the PLS and PCR models. The models were validated by testing their predictive ability on a validation set where low RMSEP, RSEP % were obtained for both models. Figures of merit were determined using the net analyte signal concept. The proposed models were applied successfully to simultaneous determination of B_1 and B_6 in presence of a low concentration of B_{12} in pharmaceutical dosage forms that contain simple excipients. The TLC densitometric method was based on the use of a developing system of chloroform: ethanol: water: acetic acid solution (2: 8: 2: 0.5 v/v) to separate the three vitamins. The separated spots were scanned at 242nm, 291nm and 360nm for B_1, B_6 and B_{12} respectively. The proposed method was applied successfully to simultaneous determination of the three vitamins in their pure powder form in the range 0.1-1.5 (µg/spot), 0.5-3.5 (µg/spot), 0.1-1.5 (µg/spot) for B_1, B_6, and B_{12} respectively and in their pharmaceutical formulations.

Keywords: Thiamine hydrochloride (B_1), Pyridoxine hydrochloride (B_6), Cyanocobalamin (B_{12}), PLS, PCR, densitometry.

INTRODUCTION

Thiamine hydrochloride (B_1), pyridoxine hydrochloride (B_6), and cyanocobalamin (B_{12}) are present together in several commercial formulations. B_1 is present in pharmaceuticals either as the hydrochloride or nitrate salt (Al-Rashood *et al*., 1989) while B_6 is used in pharmaceutical formulations as hydrochloride salt only (Aboul-Enein and Loutfy, 1984). In all these formulations, B_{12} is usually present in a concentration almost hundred times less than B_1 and B_6.

Determination of the three vitamins is described in the British Pharmacopoeia (British Pharmacopoeia, 2007) by titrimetric method for B_1 and B_6 and spectrophotometric method for B_{12}. The three vitamins were simultaneously determined by several methods including HPLC (Lebiedzinska *et al*., 2007; Li *et al*., 2004; Markopoulou *et al*., 2002; Marszal *et al*., 2005; Riccio *et al*., 2006), TLC (Bhushan and Parshad, 1994; Kartsova and Koroleva, 2007), micellar electrokinetic capillary chromatography MECC (Li and He, 1997), derivative spectrophotometry (Morelli, 1996), and mass spectrometry (Chen and Ling, 2002).

The development of spectroscopic techniques combined with multivariate calibration proved to be fast, direct and relatively less expensive methods for the simultaneous determination of drugs in mixture form in pharmaceutical formulations. Thus the research in this area is worth, looking forward for the acceptance of such methods by the regulatory agencies. Partial least squares (PLS) and principal component regression (PCR) are among the most widely applied multivariate calibration methods employed to solve data analysis problems (El-Gindy *et al*., 2006; Ghasemi and Niazi, 2005; El-Gindy *et al*., 2007; Rezae *et al*., 2005; Salem *et al*., 2003; Sena *et al*., 2004; Hadad *et al*., 2008).

Also, quantitative TLC method proved to be an inexpensive, simple way for the determination of mixtures in quality control laboratories.

The aim of this work was to develop easy, fast and sensitive methods that can be applied for the routine analysis of the three vitamins simultaneously in their pharmaceutical formulations.

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EXPERIMENTAL

Apparatus and software

Absorption measurements were done using a Labomed UVD 2950 spectrophotometer using a UVWin5.0 software. Spectral data were exported to excel and further manipulated using PLS toolbox version 2 under MATLAB 6.5 (The Mathworks, Natick, USA). All measurements were carried using 1 cm quartz cells over the range 220 – 350 nm with 1 nm interval. Camag TLC scanner, Densitometer Model 3S/N-130319, Camag muttenz Switzerland, with wincats software was used for the densitometric measurments.

Samples were spotted on precoated TLC plates, silica gel 60 F_{245} 20x 20 cm, 0.2 mm thickness (Macheray Nagel). Samples were applied to the plates using a Camag linomat V automated spray-on band applicator, equipped with a 100µl syringe

REAGENTS AND MATERIALS

Reagents used were of analytical grade. Methanol (prolabo), ethanol (prolabo), chloroform (prolabo), acetic acid (prolabo), distilled water, Ethylene diaminetetracetic acid disodium salt (EDTA) (prolabo) 1% solution in water.

Pure samples of Thaimine hydrochloride, pyridoxine hydrochloride and cyanocobalamin were kindly supplied by Glaxo SmithKline pharmaceutical company and assayed for their purity according to the British pharmacopoeial method (British Pharmacopoeia, 2007) to contain 101.0 %, 101.1% and 99.9 % respectively.

Neurovit tablets manufactured by Amriya pharmaceutical company, batch no. 7033291 labeled to contain 250 mg, 100 mg and 250 µg of B_1, B_6 and B_{12} respectively in each tablet.

Neurobion tablets manufactured by Merck, Glaxo SmithKline, batch no. 083007A labeled to contain 100 mg, 200 mg and 200 µg of B_1, B_6 and B_{12} respectively in each tablet.

Standard solutions

For the PLS and PCR methods

Stock solutions. All solutions were prepared in distilled water. Aqueous stock solutions of B_1 (1 mg/ml), B_6 (1 mg/ml) and B_{12} (0.1mg/ml).

Intermediate solutions. Appropriate volumes from the stock solutions were diluted to prepare intermediate solutions with concentrations 0.1 mg/ml for B_1 and B_6 and 0.001 mg/ml for B_{12}. These intermediate solutions were stored at room temperature in dark and were found to be stable for one month.

Calibration samples. Eleven calibration samples were prepared by diluting aliquot portions of the intermediate solutions with distilled water in 10 ml volumetric flasks to reach the final concentrations listed in table 1.

Table 1: 2^3 + three center points full factorial design for the calibration set.

Sample No.	Level		
	B_1	B_6	$B1_2$
1	0	0	0
2	-1	-1	-1
3	+1	-1	-1
4	-1	+1	-1
5	+1	+1	-1
6	0	0	0
7	-1	-1	+1
8	+1	-1	+1
9	-1	+1	+1
10	+1	+1	+1
11	0	0	0

Level (-1): 10 µg/ml B_1 , 10 µg/ml B_6 , 0.02 µg/ml B_{12}
Level (0): 25 µg/ml B_1 , 15 µg/ml B_6 , 0.11 µg/ml B_{12}
Level (+1): 40 µg/ml B_1 , 20 µg/ml B_6 , 0.2 µg/ml B_{12}

Validation samples. A validation set consisting of six standard mixtures was prepared in 10 ml volumetric flasks by mixing appropriate volumes of the three intermediate solutions and diluting to volume with distilled water (table 2). The final concentrations of B_1, B_6 and B_{12} were selected considering the calibration ranges and the concentration ratios present in the commercial samples.

Table 2: Composition of drugs in the validation set.

Sample No.	Concentration (µg/ml)		
	B_1	B_6	B_{12}
1	10.0	20.0	0.020
2	25.0	10.0	0.025
3	20.0	5.0	0.100
4	40.0	10.0	0.200
5	30.0	20.0	0.200
6	20.0	20.0	0.200

Pharmaceutical samples of neurovit and neurobion tablets. Ten tablets of each dosage form were weighed and powdered. A portion of the powder equivalent to one tablet was accurately weighed and dissolved in 100 ml distilled water in a measuring flask by the aid of magnetic stirring for 15 minutes. The solutions were filtered. One ml was transferred from each solution to a 100 ml measuring flask and completed to volume with distilled water.

For the TLC- densitometric method
Stock solutions (0.5mg/ml) of vitamin B_1, B_6 and B_{12} were prepared in methanol water (4:1) mixture

Laboratory prepared mixtures. Three lab mixtures were formed by mixing appropriate concentrations of vitamin B_1, B_6 and B_{12} pure powders in a 100 ml volumetric flask and completing the volume to 100 ml with methanol : water (4:1) mixture to prepare solutions containing 10:20:0.02, 25:10:0.025, 20:20:0.2 µg/ml of vitamin B_1:B_6:B_{12} respectively.

Procedure
For the PLS and PCR methods
Calibration set. The calibration set was prepared according to a $2^3 + 3$ (three factors at two levels with three center points) full factorial experimental design (table 1) the levels were in the concentration range 10-40 µg/ml for B_1, 10-20 µg/ml for B_6 and 0.02-0.2 µg/ml for B_{12}. The absorption spectra of the prepared solutions were measured from 220-350 nm with one nm interval. The optimized PLS and PCR models were then constructed. It should be noticed that the real samples to be analyzed do also contain excipients. The latter have been confirmed to be non-absorbent in the spectral regions of interest, and therefore there is no need for their physical separation, or for including them into the calibration set of samples.

Validation set. The absorption spectra of the validation samples were measured from 220-350 nm with one nm interval. The concentrations of B_1 and B_6 were predicted by using the proposed PLS and PCR models.

Determination of Pharmaceutical samples. The proposed models were applied to determine the concentration of B_1 and B_6 in two pharmaceutical dosage forms. The absorption spectrum for each solution was recorded from 220-350 nm and the concentration of B_1 and B_6 was determined by applying the suggested PLS and PCR models. All determinations were performed in triplicates.

For the TLC- densitometric method
Linearity. Vitamin B_1 and B_{12} solutions (0.5 mg/ml) in the range of 0.1-1.5 µg/spot and vitamin B_6 solution (0.5mg/ml) in the range of 0.5-3.5 µg/spot were applied on TLC plates previously sprayed with 1% EDTA solution and dried at 110°C. Spots were spaced 1.5 cm away from each other and from the bottom edge of the plate. The plates were developed in a chromatographic unsaturated tank using a mobile phase of chloroform: ethanol: water: acetic acid solution (2:8:2:0.5 v/v) by ascending chromatography through a distance of 15 cm at room temperature. The plates were dried then the spots were detected under the UV lamp (245 nm) then scanned under the following conditions:
measurement mode : absorption,
slit dimension : 6 x 3 mm,
scan speed :20 mm/ sec,
lamp : deuterium lamp.
wavelength : 242 nm, 291 nm and 360 nm for vitamin B_1, B_6 and B_{12} respectively,

The same procedure was used to determine the contents of vitamin B_1, B_6 and B_{12} in the three laboratory prepared mixtures and in the different batches of neurovit and neurobion tablets. The concentrations of vitamins B_1, B_6 and B_{12} were determined either by substituting in the regression equations or by comparing to standards spotted, developed and scanned under the same conditions.

RESULTS AND DISCUSSION

For the PLS and PCR methods
Spectral analysis
Fig. 1 shows the absorption spectra of B_1, B_6 and B_{12} in distilled water. A clear overlap between the spectra is observed which prevents their simultaneous determination by direct spectrophotometry. However, B_{12} is usually present in a concentration from 500 to 1000 times lower than the other two vitamins and at this ratio it does not have a considerable absorbance at the absorbance range of the other two vitamins. To overcome the overlap between B_1 and B_6 spectra, we suggested the use of simple PLS and PCR models. PLS and PCR techniques are typical full spectrum methods where the data are fit to many data points. In these techniques, a calibration model was built, validated, and then used for the prediction of concentrations of unknown samples.

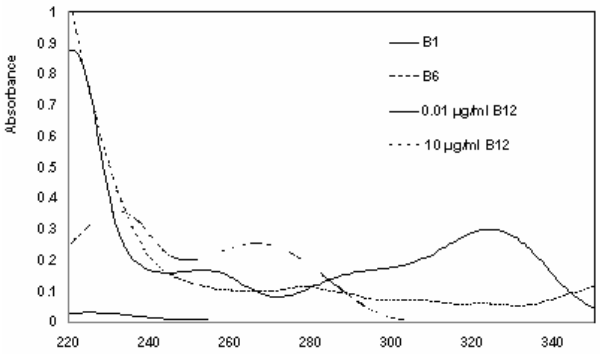

Fig. 1: Absorption spectra of 10 µg/ml B1, 10 µg/ml B6, 10 µg/ml B12 and 0.01 µg/ml B12 in distilled water.

PLS and PCR modeling
Multivariate calibration methods need the calibration set to be well experimentally designed in order to give models with good predictions. Eleven calibration samples were prepared according to a full factorial design having three factors studied at two levels with three center points (table 1). We selected the studied levels according to the concentration of the three vitamins in the available pharmaceutical dosage forms. The levels selected for B_{12} were much lower than B_1 and B_6 as it is usually present in the available pharmaceutical dosage forms in a

concentration from 500-1000 times less than the other two vitamins. Selection of the optimum number of factors for PLS and PCR methods is a very important step before building the models, as if the number of factors retained is more than required, noise will be added to the data. On the other hand, if the number of factors retained is low, meaningful data may be discarded. In this study, the cross validation method, leaving out one sample was used to select the optimum number of factors (Halland and Thomas, 1988). We compared the predicted concentrations using different numbers of factors with the known concentrations of the reference samples, and calculated the prediction error sum of squares (PRESS). The number of factors used to calculate the optimum PRESS was selected as 6 (half the number of samples + 1) (Espinosa Mansilla *et al*., 1993). A plot of the PRESS value against the number of factors for each individual component shows a minimum value for the optimal number of factors. The optimum number of factors obtained by the application of PLS and PCR was 4 factors for both B_1 and B_6 (fig. 2).

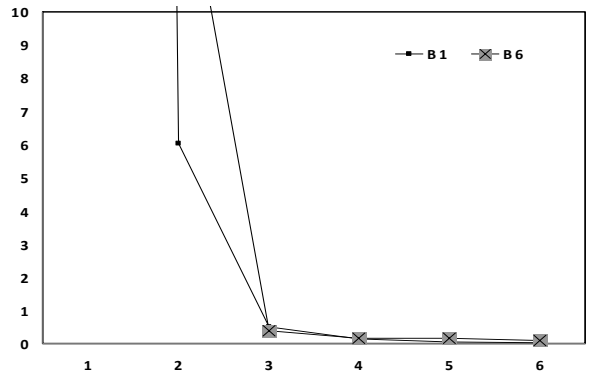

Fig. 2: PRESS plot of the crossvalidation results for the calibration set as a function of factors used to construct the calibration.

Prediction ability

To test the prediction ability of the suggested models, we used these models to predict the concentration of B_1 and B_6 in the presence of B_{12} in the validation samples. As observed from table 3, there is a good agreement between the predicted and actual concentrations of drugs. Also, to evaluate the predictive ability of a multivariate calibration model, the root mean square error of prediction (RMSEP) and relative standard error of prediction (RSEP) can be used (Ghasemi and Niazi, 2005):

$$RMSEP = \sqrt{\frac{\sum (y_{act} - y_{pred})^2}{n}} \quad (1)$$

$$RSEP(\%) = 100\sqrt{\frac{\sum (y_{act} - y_{pred})^2}{\sum (y_{pred})^2}} \quad (2)$$

where, y_{act} is the actual value of the concentration in the sample in the validation set, y_{pred} is the predicted concentration in the sample, and n is the number of samples. Results obtained are shown in table 3.

Analytical figures of merit

Figures of merit (FOM) such as selectivity, sensitivity and lower limit of detection (LOD) became very important in order to characterize, compare and develop new multivariate methods (Olivieri *et al*., 2006). Also, the determination of FOM is important for the validation of this kind of chemometric/ spectrophotometric methods (Sena *et al*., 2004). When calculating FOM for multivariate calibration methods, the part of the signal that is related only to the analyte of interest (NAS) is more important than the total signal (Booksh and Kowalsky, 1994). The definition of NAS is the part of the total signal that is orthogonal to the signal of the interferences present in the sample (Lober *et al*., 1997). The **NAS** is a vector containing the values for each sample and can be related to the regression vector, **b**, provided by the PLS or PCR models by Eq.(3) (Booksh

Table 3: Recovery percent of Predicted concentrations of vitamin B1 and B6 in the validation set obtained by the suggested PLS and PCR models

Sample No. [a]	Recovery % B_1		Recovery %B_6	
	PLS	PCR	PLS	PCR
1	102.82	102.83	100.84	100.84
2	102.00	102.01	98.32	98.30
3	102.69	102.70	99.65	99.58
4	101.43	101.44	97.66	97.73
5	100.60	100.62	99.29	99.26
6	100.08	100.11	100.97	100.95
Mean	101.60	101.62	99.46	99.44
RSD%	1.24	1.22	1.76	1.70
RMSEP	0.40	0.41	0.177	0.178
RSEP %	0.67	0.68	0.51	0.52

[a] The numbers refer to the samples in table 2.

and Kowalsky, 1994):

$$\|NAS\|_2 = \frac{1}{\|b\|_2} \quad (3)$$

where the symbol $\|\ \|_2$ means the Euclidian norm of a vector. FOM can then be calculated as functions of the **NAS** (or the regression vector) (Booksh and Kowalsky, 1994).

Sensitivity (SEN) for a given analyte is calculated as the **NAS** at unit concentration (Lober, 1986) according to Eq. (4) and the units of sensitivity are signal/concentration (Booksh and Kowalsky, 1994).

$$SEN = \|NAS\|_2 \quad (4)$$

A more indicative FOM is the analytical sensitivity (γ) (Nepote *et al*., 2003), defined by

$$\gamma = \frac{SEN}{\|\delta r\|_2} \quad (5)$$

where $\|\delta r\|_2$ is a measure of the instrumental noise. It permits the comparison of analytical methods regardless of the specific technique, equipment and scale employed (Nepote *et al*., 2003).

Selectivity (SEL) is a measure of how unique the spectrum of the analyte is compared with the other species. It ranges from 0 to 1. SEL is estimated as the ratio between SEN and the total signal (x), according to Eq.(6) (Booksh and Kowalsky, 1994):

$$SEL = \frac{\|NAS\|_2}{\|x\|_2} \quad (6)$$

Finally, for the limit of detection which is another FOM, it does not make sense to use the term limit of detection for this type of methods because the amount of analyte that can be detected is a function of the concentration of the other substance present with it (Booksh and Kowalsky, 1994). The obtained FOM are shown in table 4.

Table 4: Analytical figures of merit of the proposed PLS and PCR models.

	PLS		PCR	
	B_1	B_6	B_1	B_6
SEN*	0.14	0.13	0.14	0.13
SEL	0.35	0.33	0.35	0.33
γ	99.70	92.63	99.70	92.63
γ^{-1}	0.01	0.01	0.01	0.01
LOD μg/ml	0.03	0.03	0.03	0.03

* Absorbance units/μg

We applied the suggested models for determination of B_1 and B_6 in various dosage forms that contain low concentration of B_{12} compared to the other two vitamins. The models succeeded to determine both vitamins in two dosage forms namely, neurobion, and neurovit tablets. The corresponding results are shown in table 5. The recoveries were satisfactory as all obtained values were within 90-110%, as usually recommended by pharmacopoeias. Other dosage forms that contain more complex matrix gave predicted results more than 10% higher than those claimed on the label. This may be due to the presence of some absorbing excipients that were not modeled during calibration.

For the TLC-densitometric method

Densitometric method has been used for the determination of vitamin B_1, B_6 and B_{12} as mentioned in the literature. These methods required long procedures with many steps. In this work the attempts were made to simplify the TLC steps as to be applicable in quality control labs and routine work. Several trials were done to directly separate vitamin B_1, B_6 and B_{12} using lots of developing systems but the spots usually showed a long tail therefore the plates were sprayed with 1% EDTA solution as to chelate the calcium in them (gypsium part) producing more uniform circular fine spots. Also the development of the spots in an unsaturated chamber produced better spot shapes and separation than using a

Table 5: Determination of vitamins B_1, B_6 and B_{12} in pharmaceutical products using the proposed methods

	B_1			B_6			B_{12}
	Recovery % ± S.D.			Recovery % ± S.D.			Recovery % ± S.D.
	PLS	PCR	TLC	PLS	PCR	TLC	TLC
Neurovit Tablet	99.73 ± 1.68	99.74 ± 1.68	100.00 ± 1.46	100.43 ± 1.26	100.44 ± 1.25	99.13 ± 1.11	100.63 ± 1.65
Student's t	0.321 (2.447)			2.063 (2.447)			
F	1.322 (9.12)			1.291 (9.28)			
Neurobion Tablet	99.17 ± 1.72	99.18 ± 1.72	100.15 ± 1.66	98.82 ± 1.24	98.82 ± 1.24	98.13 ± 1.32	100.00 ± 1.47
Student's t	1.14 (2.447)			1.113 (2.447)			
F	1.074 (9.12)			1.125 (9.28)			

N.B: Figures between parenthesis are the corresponding tabulated values (p = 0.05)

Table 6: Results of assay validation obtained by applying the proposed TLC method

Parameter	Vitamin B_1	Vitamin B_6	Vitamin B_{12}
Accuracy Mean ± S.D	99.84 ± 1.25	99.79 ± 1.13	99.61 ± 1.46
Precision			
Interday [a]	1.901	1.594	1.996
Intraday [a]	1.793	1.306	1.729
Specificity			
Student's t	0.436 (2.145)	1.716 (2.145)	0.581 (2.145)
F	2.039 (4.15)	1.625 (3.58)	1.373 (4.15)
LOD[b]	0.05 µg.spot^{-1}	0.3 µg.spot^{-1}	0.05 µg.spot^{-1}
LOQ[b]	0.1µg.spot^{-1}	0.5µg.spot^{-1}	0.1µg.spot^{-1}
Linearity			
Slope	Coefficient 1:-2051 Coefficient 2:7516	1785.4	Coefficient 1:-3199 Coefficient 2:1468
S.E. of Slope	S.E. of Coef.1:150.6 S.E. of Coef.2:244.8	16.1	S.E. of Coef.1 :233.2 S.E. of Coef.2:379
Intercept	-189.6	114.6	-1137.7
S.E. of Intercept	79.5	36	123
Correlation coef.	0.9993	0.9995	0.9996
Range	0.1-1.5 (µg/spot)	0.5-3.5 (µg/spot)	0.1-1.5 (µg/spot)

N.B: Figures between parenthesis are the corresponding tabulated values (p = 0.05), [a]n = 12, [b] Determined practically

saturated one. The best separation of spots was obtained upon using of ethanol: water: chloroform: acetic acid solution (8:2:2:0.5 v/v) as a developing system where vitamin B_1, B_6 and B_{12} showed R_f values of 0.15, 0.75 and 0.45 respectively. The separated spots were scanned at 242 nm, 291 nm and 360 nm for vitamin B_1, B_6 and B_{12} respectively. We obtained a linear correlation between the peak area of the separated spots and the corresponding concentrations of vitamin B_6 in the range of 0.5-3.5 µg.spot^{-1} from which the linear regression equation was calculated (table 6).

For vitamins B_1 and B_{12} the relationship between the peak area of the separated spots and the corresponding concentrations showed bad correlation coefficient upon applying the linear regression fit. By examining the residual analysis it confirmed that the linear fit should be rejected. The polynomial fit was investigated and found to be better than the linear fit, and the residuals showed good scatter around zero. So the relation between the concentration of both vitamins and the obtained peak areas was expressed as second order polynomial equations in the range of 0.1-1.5 µg.spot^{-1} for both vitamins (table 6).

By applying the proposed densitometric method, it was possible to determine the three vitamins in their pure powder form, where good recoveries were obtained. To assess accuracy and precision of the proposed method, a series of laboratory prepared mixtures were analyzed by the proposed procedure. The results obtained proved the good performance of the method and the recoveries were found to be 99.66 ± 1.79, 98.89 ± 1.22 and 99.89 ± 1.71 for B_1, B_6 and B_{12} respectively. Different parameters for the validation of the TLC method were calculated, (Table 6). The proposed procedure was successfully applied for the analysis of vitamin B_1, B_6 and B_{12} in their pharmaceutical dosage form. No interference due to excipients was detected and the results of analysis in table 5 indicates that the proposed method can be used for the quantitative determination of vitamin B_1, B_6 and B_{12} in their tablet form.

Results obtained by applying the proposed TLC, densitometric method were statistically compared with those obtained from the PLS method .Table 5 showed that the calculated t and F values were less than the theoretical ones, indicating that the densitometric and PLS methods have the same accuracy and precision at 95% confidence level.

In spite of that the PLS and PCR methods are more simple and less time consuming, the densitometric method has the advantage of being able to determine all the three vitamins at any ratio.

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