Development of TLC- Densitometeric Method Used for Quantitative Estimation of Some Natural Pharmaceutical Preparations in Egyptian Market

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Abstract: Phytopharmaceuticals and dietary supplements are launched into the market without proper scientific evaluation. In this study, TLC-densitometric method was developed for the quantitative estimation of L-Carnitine capsules and syrup, Kellagon capsules which contain (*Ammi visnaga* and *Cymbopogon proximus* extracts) in Egyptian market. L-carnitine R_f was 0.47 using mobile phase: chloroform: methanol: formic acid: water [65:65:4:4 v/v/v/v] with double development, spots intensities were measured by densitometry at λmax = 500 nm. The assay of L-Carnitine capsules was 383.25 mg/capsule (109.5%) and of L-Carnitine syrup was 289.5 mg/ml syrup (96.5%). Both khellin and proximadiol was chosen as markers for quantitative estimation of *Ammi visnaga* and *Cymbopogon proximus* extracts respectively. Khellin R_f was 0.39 using mobile phase: chloroform: methanol [99:1 v/v] and the spots were measured at λmax = 440 nm. Proximadiol R_f was 0.18 using mobile phase: chloroform: methanol [95:5 v/v], measured at λmax = 530 nm. The assay of Kellagon capsules yielded 6.6 mg khellin /Capsule and 0.44 mg proximadiol /Capsule.
The method was validated and showed accuracy, selectivity and precision, hence can be used for a routine quality-control analysis and simultaneous quantitative determinations.
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Key words: Khellin, L-carnitine, proximadiol, TLC-Densitometer

1. Introduction

Medicinal plants constitute a source of raw materials for both traditional systems of medicine (e.g. Ayurvedic, Chinese, Unani, Homeopathy, and Siddha) and modern medicine. Nowadays, plant materials are employed throughout the industrialized and developing world as home remedies, over-the-counter drugs, and ingredients for the pharmaceutical industry.

Phytopharmaceuticals are always mixtures of many constituents and are therefore very variable and difficult to characterize. The active principle(s) in phytopharmaceuticals are not always known.

In most countries herbal products and dietary supplements are launched into the market without proper scientific evaluation while consumers can buy herbal products without a prescription and one might not recognize the potential hazards in an inferior product.

Quality control for the efficacy and safety of products containing natural compounds becomes essential (**Farnsworth *et al.*, 1985; WHO, 1990, 1999; Pal and Shukla, 2003**).

L-carnitine (L-3-hydroxy-4-aminobutyrobetaine), and its acyl esters (acylcarnitines) are essential compounds for the metabolism of fatty acids. They are present in animals, plants and some microorganisms. In animal tissues, carnitine concentrations are relatively high, typically between 0.2 and 6 mmol/kg, with most in the heart and skeletal muscle (**Bourdin *et al.*, 2007**). The main function of carnitine in the body is to facilitate lipid oxidation by transporting long-chain fatty acids into the inner mitochondria region where they undergo β-oxidation (**Bieber, 1998**). In order for fatty acids (from food intake or adipose tissue) to produce energy they must be changed into acylCoAs prior to β-oxidation; however, since acylCoAs can not cross cell walls, carnitine comes into place to help with the transportation through the mitochondrial wall. Therefore, without carnitine, most of the dietary lipids cannot be used as an energy source and the body would accumulate fatty-acids resulting in obesity (**Hamilton *et al.*, 1983; Borum, 1986; Cha, 2008**).

L-carnitine administration has been proven to be clinically beneficial in diseases characterized by carnitine deficiency, which include ischemic cardiomyopathy and peripheral atherosclerosis.

L-carnitine supplementation was found to improve the quality of life in terms of improvement in physical performance and various co morbid conditions, like weakness, poor exercise tolerance and easy fatigability. Scattered reports indicate that supplementation of L-carnitine will improve patient status and symptoms, with significant improvement in laboratory parameters, especially on lipid levels as well as inflammatory and nutritional parameters among heart diseases patients (**Suchitra *et al.*, 2011**).

Ammi Visnaga L. (Apiaceae), fruits is much used by the population as a diuretic and antispasmodic in cases of ureteral stones. Of the several crystalline substances isolated from the seeds, quantitatively the most important is a furanochromone known as khellin

which recently used as a phototheraputic agent against psoriasis and vitiligo (**Mawatari *et al.*, 2003**).

Cymbopogon proximus STAPF. (Gramineae) is a weed known as Halfabar that grows in the Egyptian desert. It is highly reputed in Egyptian folk medicine as an effective renal antispasmodic and diuretic agent. Proximadiol is the most important active principle (**El-Askary *et al.*, 2003**).

The purpose of this work is to develop TLC methods for quantitative estimation of some drugs containing natural compounds available in Egyptian market, L-Carnitine capsules and syrup, Kellagon capsules (mix of *Ammi visnaga* and *Cymbopogon proximus* extracts), as there is no simple method reported for their quantitative determination**.**

2. Material and Methods

2.1 Materials

Khellin and L-carnitine working standards were purchased from Sigma-Aldrich. Proximadiol were obtained as a gift sample from National Research Center, Egypt. The formulation, L-Carnitine capsules (350 mg/capsule), L-Carnitine syrup (300 mg/ml) and Kellagon capsules (mix of *Ammi visnaga* extract (180 mg) and *Cymbopogon proximus* extract (72 mg)) available in the Egyptian market manufactured by Mepaco company; chloroform, formic acid, butanol, methanol and glacial acetic acid were of analytical grade, pre-coated silica gel 60 F_{254} TLC plates (Merck). All dilutions were performed in standard volumetric flasks.

2.2 Methods

2.2.1 Preparation of standard solutions:

L- carnitine standard stock solution was prepared in a concentration 20 mg/mL with methanol. Serial dilutions were prepared to obtain 4, 6, 8, 10, 12, 14 , and 16 mg/ml for construction of the calibration curve.

Khellin standard stock solution was prepared in a concentration 20 mg/mL with methanol. Serial dilutions were done to obtain 1, 2, 4, 6, 8, 10 and 12 mg/ml for construction of the standard calibration curve.

Proximadiol standard stock solution was prepared in a concentration 100 mg/ml with methanol. Serial dilutions of the stock solution were prepared to obtain 2, 4, 6, 8 and 10 mg/ml for construction of the standard calibration curve.

2.2.2 Preparation of sample solutions:

For L-Carnitine capsules the content of three capsules were weighed. The average weight was calculated and a quantity equivalent to one capsule was accurately weighed and dissolved in methanol into a 10 ml volumetric flask using a Sonicator for about 30 minutes, diluted up to the mark with methanol and mixed well. One ml was taken and diluted with methanol to 5 ml in 5ml volumetric flask to give a final concentration 7 mg/ml.

For Carnitine syrup 1ml of the solution was diluted to 10 ml in a volumetric flask, and then one ml was diluted with methanol to 5 ml in 5 ml volumetric flask to give a final concentration 6 mg/ml.

The content of six kellagon capsules were weighed and the average weight was calculated and a quantity equivalent to three capsules was accurately weighed and dissolved in methanol into a 10 ml volumetric flask and the volume was made up to 10 ml with methanol.

2.2.3 Chromatographic conditions:

L-carnitine Stationary Phase: Silica gel 60 F_{254} plates, Mobile phase: chloroform: methanol: formic acid: water [65:65:4:4 v/v/v/v] with double development. Spray reagent: 1% ninhydrin spray reagent gives red coloured spots.

Khellin Stationary Phase: Silica gel 60 F_{254} plates, Mobile phase: chloroform: methanol [99:1 v/v] with single development. Spray reagent: p-anisaldehyde spray reagent gives yellow coloured spots.

Proximadiol Stationary Phase: Silica gel 60 F_{254} plates, Mobile phase: chloroform: methanol [95:5 v/v] with single development. Spray reagent: p-anisaldehyde spray reagent gives violet coloured spots.

2.2.4 Development of TLC Technique

The samples were spotted (10 µL) in the form of 8mm bands using a micropipette on a precoated silica gel plates 60 F_{254} [10 cm X 10 cm with 0.2 mm thickness, E. Merck]. The plates were developed in a solvent system in glass twin through chamber previously saturated with the solvent for 30 min. the distance was 8 cm. Prior to scanning, TLC plates were air dried, sprayed with specific spray reagent for each analyzed compound, heated at 160°C for three minutes. Scanning was performed on Shimadzu TLC flying spot scanning densitometer (Japan). The chromatographic conditions had previously been optimized to achieve the best resolution and peak shape. Plates were evaluated by densitometry at λmax = 500 nm for L-carnitine, λmax = 440 nm for khellin, and λmax = 530 nm for proximadiol.

2.2.5 Assay (for pharmaceutical preparations)

10 µL working standard solution (70 µg/spot of L-carnitine, 20 µg/spot of khellin and 10 µg/spot of proximadiol) and sample solutions were spotted on the plate. The plate was developed and evaluated as described above. The procedure was repeated three times, individually weighing the capsule powder each time. The densitometric responses from both the standard and sample were used to calculate the amounts of the drug/extract in the capsules. The results obtained are as shown in Tables 1a&1b

2.2.6 Validation Procedures

2.2.6.1 Linearity

Different concentrations of each working standards were prepared from stock solutions in the range of 2 to 20 mg/mL for L-carnitine, 1 to 14 mg/ml for khellin, and 1 to 12 mg/ml for proximadiol in methanol to obtain the desired linearity range; 10 µL of each solution were applied to a plate using a micropipette and the plate was developed. The detector response to the different concentrations was measured. The standard peak-area was calculated for each concentration level. A graph of drug concentration against the peak area was plotted; the data obtained are given in Table 2.

2.2.6.2 Limit of detection and limit of quantitation

The limit of detection (LOD) and Limits of quantitation (LOQ) from the proposed method were determined using calibration standard. LOD was calculated as 3 σ/S and LOQ was calculated as 10 σ//S where S is the slope of the calibration curve and σ is the standard deviation of Y- intercept of regression equation . (**El-Bagary RI *et al*., 2011**).

3. Results and Discussion

Different compositions of the mobile phase for TLC analysis were tested to obtain high resolution and reproducible peaks. This was achieved using chloroform: methanol: formic acid: water 65:65:04:04 with double development for L-carnitine, chloroform: methanol 99:01 for khellin and chloroform: methanol 95:05 for proximadiol. Both khellin and proximadiol were chosen as markers for quantitative estimation of *Ammi visnaga* and *Cymbopogon proximus* extracts in Kellagon capsules.

L-Carnitine capsules and syrups showed single peak R_f value 0.47 coinciding with R_f value of standard carnitine.

Kellagon capsule showed 13 peaks, the ninth peak R_f value 0.39 and twelfth peak R_f value 0.18 were coinciding with R_f value of standard khellin, and proximadiol respectively.

Figures (1-3) shows the typical densitograms obtained from L-carnitine, khellin and proximadiol respectively from which it is clear that peak areas is concentration dependent.

The correlation of coefficient (r^2) from the standard calibration curves was 0.9900 for L-Carnitine, 0.9953 for khellin, and 0.9955 for proximadiol. This means that a good linear relationship was observed between the concentration range 40 to 160 µg/spot, 10 to 120 µg/spot and 20 to 100 µg/spot for L-carnitine, khellin and proximadiol respectively.

The limit of detection (LOD) was found to be 0.46µg/spot for L-carnitine, 4.4µg/spot for khellin, and 0.1µg/spot for proximadiol. The LOQ was found to be 4.6 µg/spot for L-carnitine, 14.8 µg/spot for khellin, and 0.35µg/spot for proximadiol. These values indicate adequate sensitivity of the method.

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The accuracy and precision from recovery analysis for L-carnitine, khellin and proximadiol are listed in Tables 3, 4 and 5 respectively.

The assay of L-Carnitine capsules was 383.25 mg/capsule (109.5%) and of L-Carnitine syrup was 289.5 mg/ml syrup (96.5%), while khellin concentration was 6.6 mg /Capsule and proximadiol concentration was 0.44 mg/Capsule, indicating good recovery for the proposed method.

Table 1a: Results of TLC assay studies (from Carnitine capsule & Carnitine syrup) (n=3)

		% CV	% Recovery	Amount found
Carnitine capsule	350 mg	1.9	109.5	383.25 mg
Carnitine syrup	300 mg	2.0	96.5	289.5 mg

Table 1b: Results of TLC assay study (from Kellagon capsule) (n=3)

	% CV	Amount found
Khellin	0.02	6.6 mg /Capsule
Proximadiol	0.07	0.44 mg/Capsule

Table 2: Linear regression data for calibration curves

	L-carnitine	Khellin	Proximadiol
Linearity	40 to 160 µg/spot	10 to 120 µg/spot	20 to 100 µg/spot
Correlation coefficient	0.9900	0.9953	0.9955

Table 3: Results from recovery analysis for carnitine (n=3)

Concentration (mg/ml)	%CV (Precision)	Accuracy
4	6	109
6	3	99.8
8	6	108.9
10	7	110
12	5.3	107
14	8.7	115
16	8.6	116

Table 4: Results from recovery analysis for khellin (n=3)

Concentration	%CV (Precision)	Accuracy
1	6.3	95
2	3.1	98.2
4	6.6	96
6	0.21	100.2
8	7.3	95
10	5.1	96.5
12	2.6	98.5

Table 5: Results from recovery analysis for proximadiol (n=3)

Concentration	%CV (Precision)	Accuracy
2	10.5	107
4	10.4	108
6	10.9	92
8	6.7	103
10	6.1	93

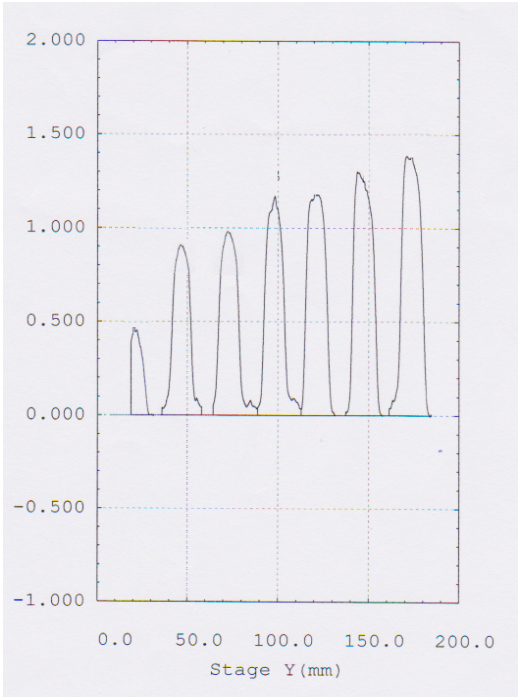

Fig 1.TLC chromatogram of L-carnitine standard.

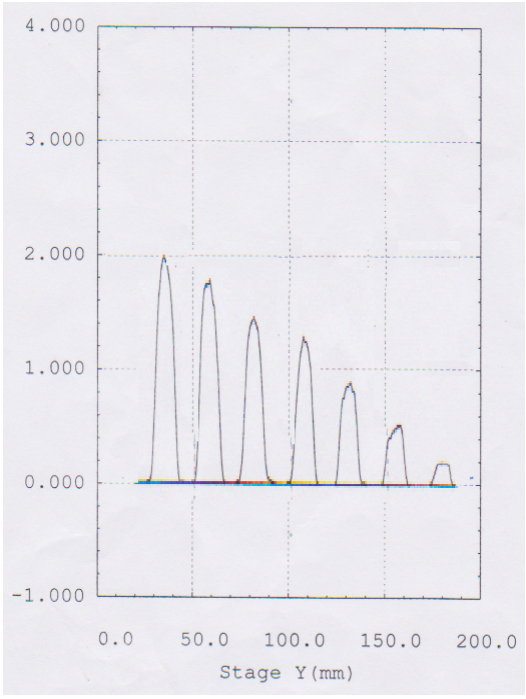

Fig 2. TLC chromatogram of khellin standard.

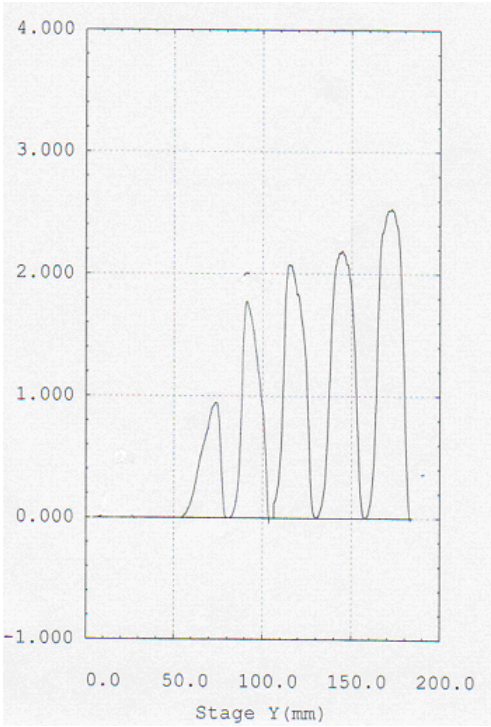

Fig 3. TLC chromatogram of proximadiol standard

Conclusion

As the proposed method is accurate, selective and precise hence can be used for a routine quality-control analysis and simultaneous quantitative determination of L-Carnitine capsules and syrups, and for preparations containing *Ammi Visnaga* and *Cymbopogon proximus* extracts using khellin and proximadiol as markers.

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