A Simple and Rapid HPLC-UV Method for the Determination of Umckalin, as an Herbal Marker, in the Cough Syrup of Pelargonium Extract

M. Hudaib [1]✉, W. B. Aldahasi[2], A. Khdair[3], R. Salah[4], H. S. AlKhatib[1], Y. Bustanji[1] and M. K. Mohammad[1]

[1] Faculty of Pharmacy, University of Jordan, Amman, Jordan
[2] Biovision Company, Riyadh, Saudi Arabia
[3] Faculty of Pharmacy, Applied Sciences University, Amman, Jordan
[4] Sana Pharmaceutical Research Co., Amman, Jordan

ABSTRACT

A rapid and simple high performance liquid chromatography (HPLC) method with UV detection for the quantitation of umckalin, as an herbal marker, in Pelargonium extract cough syrup has been developed and validated. Chromatographic separation was achieved on a reverse phase Phenomenex®-C_{18} column (5 µm, 25 cm × 0.5 mm i.d.) using a mixture of acetonitrile and phosphoric acid (pH 2.5), in 25:75 (v/v) ratio, as a mobile phase at a flow rate of 1 mL/min under ambient conditions and with UV detection at 310 nm. The method, applied for umckalin quantitation, showed good linearity over the concentration range of 0.334 –1.667 µg/mL, with a correlation coefficient (r^2) of 0.9996. The limit of detection (LOD) and limit of quantitation (LOQ) of umckalin were found to be 0.0344 and 0.1031 µg/mL, respectively. In addition, the developed HPLC method showed acceptable values of repeatability and intermediate precision and indicated high levels of method accuracy. Simplicity and validity of the method make it highly reliable and especially suitable for routine quality control analysis.

Keywords: Umckalin, *Pelargonium sidoides*, HPLC analysis, Cough syrup, Herbal product, Herbal marker.

INTRODUCTION

Pelargonium sidoides DC. (family: Geraniaceae) is a medicinal plant, with woody roots, that has been used for the treatment of upper and lower respiratory tract diseases for hundreds of years[1,2]. Moreover, antibacterial and immunomodulatory activities have been proven[3,4]. Several commercially available extracts of *P. sidoides* have clinically and pharmacologically proven efficacy for the treatment of cough associated with acute bronchitis or common cold[5,6]. Such extracts are commonly called "Umckaloabo". For example, EPs® 7630 (Umckaloabo®, Spitzner Arzneimittel) has been developed from extract of *P. sidoides* and introduced into the European market[7].

Received on 22/11/2009 and Accepted for Publication on 14/3/2010.

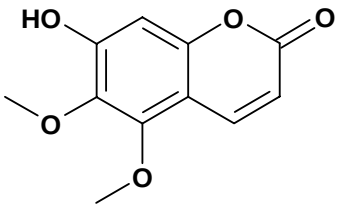
✉ E-mail: m.hudaib@ju.edu.jo

Phytochemically, previous reports have led to the characterization of several metabolites including phenolic and cinnamic acids, tannins, flavonoids, and coumarins in the different parts of *P. sidoides*[1,8]. The plant extracts, obtained particularly from roots, were found to contain substituted and unsubstituted oligomeric prodelphinidins, monomeric and oligomeric carbohydrates, minerals, peptides, purine derivatives, and highly substituted benzopyranones, which include mainly umckalin (7-Hydroxy-5,6-dimethoxycoumarin) as the most common bioactive component of this plant species. Nearly 230 components have been also detected in the plant essential oil, with sesquiterpenes the most abundant[9].

Despite a wide pharmaceutical use of *P. sidoides*, erroneous identification of the raw plant material and confusion with other species especially *P. reniforme* is problematic to the manufacturing and use of the

plant[10,11]. In addition, quantitative analysis in the quality control of finished pharmaceutical products is a burden to the manufacturing of such products. Thus, precise analytical methods to identify *P. sidoides* in raw materials and more importantly in the pharmaceutical products are needed to enable safe and effective use of the plant. Previous studies have reported identification and analysis of *P. sidoides* of wild harvested and cultivated plants using HPLC[12]. However, up to our knowledge there is no previous method available for quantitative analysis of *P. sidoides* in pharmaceutical products. In the present work we report the development of a simple, sensitive, precise, and specific HPLC method for the analysis of a *P. sidoides*-based pharmaceutical product (syrup).

The method reported here is an isocratic reversed phase HPLC assay with UV detection that can be applied to the determination of umckalin in commercially available pharmaceutical products of *P. sidoides*. Umckalin, the bioactive constituent typical to *P. sidoides*, is used as a reliable identifying marker[3,8].



Umckalin

EXPERIMENTAL

Materials and Instruments

Acetonitrile and phosphoric acid were purchased from Fischer Scientific (Chicago, IL). Pelargonium dried extract (standardized to contain 330 ppm umckalin), authentic umckalin reference standard, as well as the Pelargonium syrup (potency: 5 µg/mL umckalin) were a gift from SANA Pharmaceutical Research Co. (Amman, Jordan).

A Finnigan™ HPLC system with Surveyor® LC Pump Plus and Surveyor® Autosampler Plus connected to Surveyor® PDA Plus detector was used (Thermo Electron Corporation, San Jose, CA). The column was a reverse phase Phenomenex®-C_{18} (5 µm, 25 cm × 0.5 mm i.d., Thermo Electron Company, Bellefonte, North America). Data from each chromatographic run were processed using ChromQuest® v 4.1 LC data system (Thermo Electron Corporation, USA).

Methods

Preparation of Standard Solutions and Calibration Curve

A standard stock solution was prepared by transferring accurately weighed 2.50 mg umckalin reference standard into a 50 mL volumetric flask. Then 40 mL of the diluent (the mobile phase) was added followed by sonication for 15 minutes or until dissolved. The volume was completed with the same diluent and then filtered through 0.2 µm membrane filter.

To study the linearity range of umckalin, serial dilutions of the above standard stock solution were made to prepare the calibration solutions, which were analyzed by HPLC as described below. A graph was plotted as concentration of drug versus peak area (response) and found linear in the range of 0.334-1.667 µg/mL of umckalin.

Preparation of Samples

Four milliliters of the liquid samples (Pelargonium syrup or diluted extract) were mixed with 15 mL of the mobile phase and then sonicated for 15 min. Volume was adjusted to 20 mL with the mobile phase and processed as described under the standard solution preparation. For purpose of product assay, three quality control sample solutions were prepared according to this procedure with a supposed final concentration of 1 µg/mL umckalin (test concentration). The prepared solutions were analyzed, twice each, by the developed HPLC method. Further samples were also prepared for precision assessment as will be described below.

HPLC Method

Twenty µL aliquots of standard and sample solutions were injected in triplicate into the column and umckalin was detected at 310 nm wavelength. A mobile phase consisting of filtered and degassed mixture of acetonitrile and phosphoric acid (pH 2.5) in 25: 75 (v/v) ratios was

used at a flow rate of 1 mL/min under ambient conditions (25 °C). Under these conditions the peak corresponding to umckalin was identified at 11.13 min average retention time.

Method Validation

The HPLC method was validated in terms of linearity, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ), and accuracy according to the USP pharmacopoeia and International Conference on Harmonization (ICH) guidelines[13]. Precision of the assay method was determined using six-independent test solutions at concentration of 1 µg/mL. Linearity of the assay was determined over five different umckalin concentrations (0.334, 0.667, 1.000, 1.334, and 1.667 µg/mL), each analyzed in duplicate. The concentrations of calibration solutions, used in linearity assessment, were chosen to cover the specified range of the method (50%-150% of the test concentration). The response, represented by the average peak area, was plotted against concentration and least square regression analysis was applied. LOD and LOQ of the method were calculated using the slope (S) of the calibration curve, obtained from linearity assessment, and the standard deviation of the response (SD) calculated as the standard deviation of the y-intercepts of regression lines plotted at the chromatographic response values. Specificity of the assay method was evaluated by injecting Pelargonium syrup placebo, consisting of all matrix components with the exception of the dried extract, in duplicate, under the analytical conditions of the developed method. Precision of the method was evaluated by repeatability (intraday) and intermediate precision (interday) tests and evaluated by the calculated coefficient of variation (CV), or relative standard deviation (RSD), where values of less than 2% were considered acceptable. The method accuracy was evaluated as the percent recovery of the added standard from the accuracy samples, which were prepared by the addition (spiking) of known amounts of umckalin reference standard to the placebo. Accuracy was evaluated at five different concentrations (0.333, 0.666, 1.000, 1.333 and 1.666 µg/mL) of umckalin, analyzed in duplicate.

RESULTS AND DISCUSSION

In the present study a reversed phase HPLC-UV method for the separation and quantitation of umckalin in a *P. dioides* extract containing pharmaceutical syrup was developed. The chromatographic conditions were adjusted and optimized to provide reliable and efficient chromatographic and assay performances. In particular, the selection of mobile phase was based on peak parameters, ease of preparation, cost, and total analysis time. In the best scenario, separation of umckalin from other matrix components was successfully achieved in about 12 min total analysis time as shown in Fig. 1.

Method Validation

Specificity

According to ICH guidelines and some other compendial sources, for chromatographic procedures, specificity is usually demonstrated by representative chromatograms where individual components should be appropriately labeled.

In order to determine the specificity of the present HPLC method, the chromatogram obtained from placebo analysis (Fig. 1A) was compared with the one obtained after Pelargonium extract syrup analysis (Fig. 1B). As shown, a complete separation of the umckalin peak was demonstrated without any interference by any other syrup component at the retention time of the drug in the chromatogram of placebo solution. Moreover, in a peak purity analysis, performed using a photo diode array detector, no peaks appeared at the retention time of umckalin with a calculated peak purity of 0.9965.

Linearity

Linearity of an analytical method is defined as its ability to give test results or a response that is proportional to the concentration of analyte in specified range either directly or by mathematical transformation[13]. The ICH guidelines indicate that a specified range is usually determined according to the purpose of the analytical method. For example, the assay of an active ingredient is usually tested in the range of 80%-120% of test concentration. In our experiment, the selected range was 50%-150% of the test concentration (1

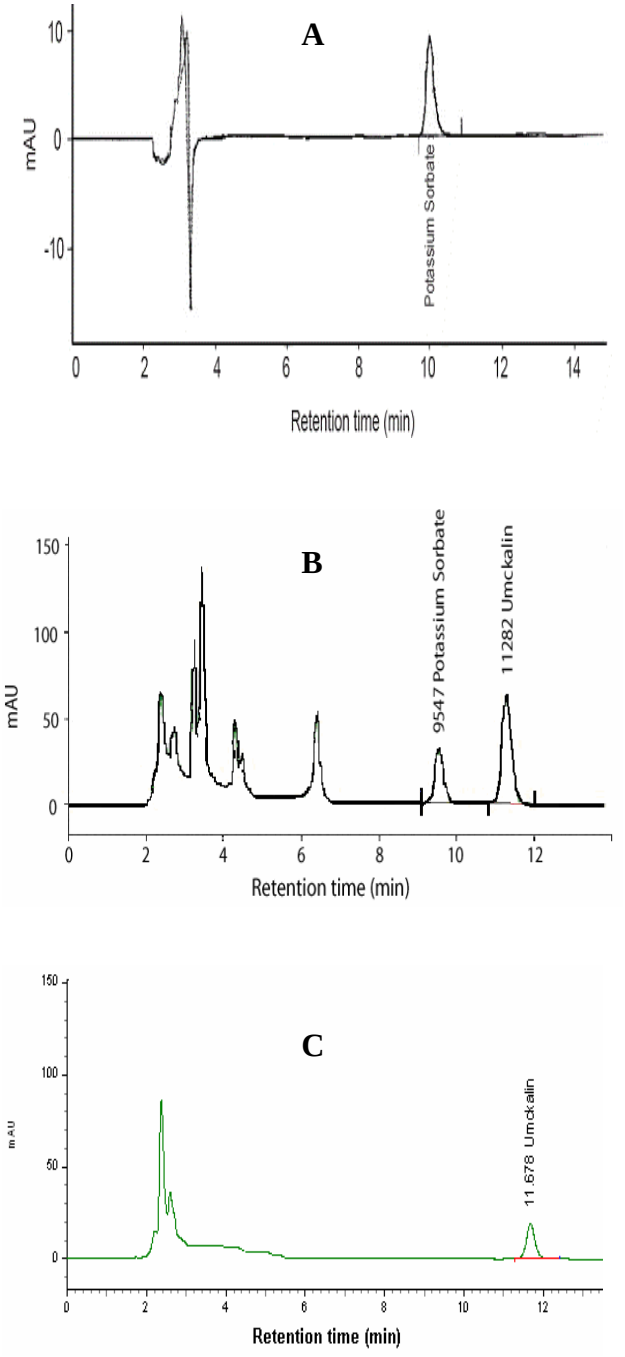


Figure 1: HPLC chromatogram of **(A)** Pelargonium extract syrup placebo, **(B)** Pelargonium extract syrup, and **(C)** Umckalin standard.

µg/mL), which corresponded to a range of (0.5-1.5) µg/mL of umckalin concentration.

As recommended by the ICH guidelines, the linearity of an analytical method should be determined at least by five different concentrations covering the specified range. As shown in Figure 2, linearity was obtained over the range of 33.4-166.7% of test umckalin at five different concentrations (0.334, 0.667, 1.000, 1.334, and 1.667 µg/mL). The Average regression equation over the range of 0.334 –1.667 µg/mL was y = 882115x -13175 with a slope of 882115, an intercept of 13175 and a correlation coefficient (r^2) value of 0.9996.

LOD and LOQ

Several approaches are proposed by the ICH guidelines to determine LOD and LOQ of instrumental analytical procedures. In the present study these values were determined as follows: LOD = 3.3×SD/S, LOQ = 10×SD/S (or LOQ = 3×LOD).

Where, SD = the standard deviation of the response, and S = the slope of the calibration curve. The slope (S) was estimated from the linear regression equation described in the previous section. Particularly, the value of SD was estimated as the standard deviation of the y-intercepts of 5 regression lines, each of which is plotted parallel to the calibration line and passing through the average chromatographic response of a given calibration standard. The SD value was further confirmed by another method based on the residual standard deviation of the calibration line (data are not given). The values corresponding to LOD (0.0344 µg/mL), and LOQ (0.1031 µg/mL) and the method used for their calculation are all summarized in Table 1. Noteworthy, the obtained LOQ value was lower than the lowest value of the linearity range. Subsequently, the LOQ was validated by the independent analysis, in duplicate; of 6 samples containing umckalin in concentration very close to the quantitation limit (data are not given).

As shown (Table 1), the results indicated that the current method is suitable to detect and measure very small product concentrations of umckalin.

Precision

Precision can be defined as the measurement of

degree of agreement for a set of test results after repeatable sampling from a homogenous sample. It is usually measured using relative standard deviation (RSD) or coefficient of variation (CV) with accepted values to be less than 2%. The precision of the current assay method was determined by repeatability (intraday precision) and intermediate (interday) precision. Repeatability was evaluated by assaying 6 sample solutions, prepared at 100% level of the test content (1.0 µg/mL), during the same day. The intermediate precision was assessed by comparing the results of repeated assays performed in another day (24 hours apart).

The data of repeatability and intermediate precision investigations, presented in Table 2, demonstrated that the current method can be used for umckalin analysis with acceptable level of precision.

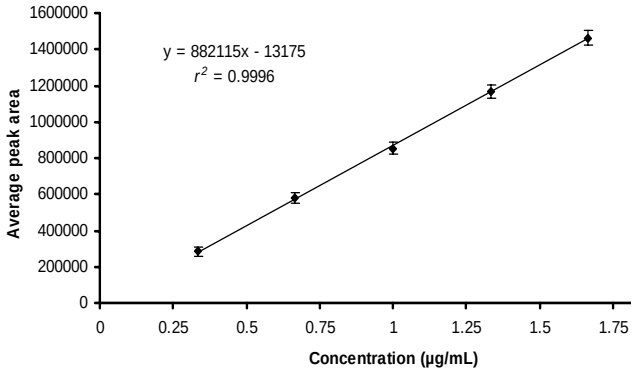


Figure 2: Linearity of umckalin response (peak area) over the concentration range of 0.334-1.667 µg/mL (the y-error bars represent the standard deviation of the response at each concentration).

Accuracy

Accuracy could be defined as the closeness of test results obtained by the analytical method to the true value. According to ICH guidelines, the accuracy of an analytical method should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range of the method[13]. The recovery percentage for a herbal preparation is considered to be generally acceptable if it lies within 95%-106% of the true value.

In the present study, accuracy was determined at five different concentration levels (0.333, 0.666, 1.000, 1.333 and 1.666 µg/mL) of umckalin, in duplicate, to allow the calculation of percent recovery values. As shown in Table 3, the accuracy results, expressed as mean percent recovery (± RSD), proved that the developed method is accurate.

Product Assay

The assay results of quality control sample solutions analyzed by the developed method showed an average content of 99.3% (RSD = 0.8%) of labeled umckalin amount in the tested Pelargonium syrup.

CONCLUSIONS

In this study, a reversed-phase HPLC method for the analysis of pharmaceutical products (syrups) based on extracts from *P. sidoides* has been developed and validated for the quantitative determination of the reference marker, umckalin. Validation was performed according to the validation protocol of ICH guidelines, which showed that the developed HPLC assay is simple, specific, linear, precise, and accurate. The reported method was very specific as the peak corresponding to umckalin, the marker, was well separated from the peaks of other product components (impurities and excipients) with a total runtime of 12 min. The method was found linear over the concentration range of 0.334-1.667 µg/mL. In addition, the current method showed also high levels of precision, repeatability, and intermediate precision and indicated high levels of method accuracy. Simplicity and validity of the method make it highly reliable and especially suitable for routine quality control and analysis.

ACKNOWLEDGEMENT

The authors wish to thank the Deanship of Academic Research (DAR) at the University of Jordan for their generous funding of this project.

Table 1: Calculation and results of limit of detection (LOD) and limit of quantitation (LOQ).

Concentration (µg/mL)	y-intercept*	Standard deviation of the response (SD)	Limit of detection (LOD)[a]	Limit of quantitation (LOQ)[a]
0.334	-9239.41	9183.794	0.0344 µg/mL	0.1031 µg/mL
0.667	-8010.71			
1.000	-29227.00			
1.334	-12285.40			
1.667	-7112.71			

* At each concentration, the y-intercept was calculated from linear regression equation (y = 882115x – 13175) as follows: y-intercept = y – 882115x, where y: is the average peak area (response) obtained at each concentration.

[a] For calculation of LOD and LOQ, see Experimental.

Table 2: Repeatability and intermediate precision tests: Chromatographic peak areas and retention times of umckalin in Pelargonium extract syrup samples (n = 6) made at a concentration of 1µg/mL.

Sample number	1	2	3	4	5	6	RSD*
Measured peak area (Day 1)	866416	864915	871242	870642	853580	857345	0.828
Retention time	11.14	11.14	11.13	11.13	11.12	11.12	0.08
Measured peak area (Day 2)	873345	870332	868762	874305	865947	868562	0.362
Difference (%)	0.8	0.63	0.28	0.42	1.45	1.31	NA

RSD: relative standard deviation; * Values of less than 2% are usually acceptable.

Table 3: Accuracy study: Mean percent recoveries of umckalin from placebo samples spiked with known amounts of umckalin reference standard and analyzed by the developed HPLC method.

Percent of the labeled umckalin content (1µg/mL)	Added amount of umckalin (µg)	Found amount of umckalin (µg)	% recovery*
Level 1 (33.3%)	0.333	0.348	105.3
	0.333	0.353	
Level 2 (66.6%)	0.666	0.693	103.9
	0.666	0.691	
Level 3 (100.0%)	1.000	1.007	100.6
	1.000	1.005	
Level 4 (133.3%)	1.333	1.379	103.5
	1.333	1.381	
Level 5 (166.6%)	1.666	1.716	102.4
	1.666	1.696	
Mean % recovery ± RSD			103.1 ± 1.7

* The average value of two determinations; RSD: Relative standard deviation

REFERENCES

(1) Kolodziej, H. In: Geranium and pelargonium: the genera *Geranium* and *Pelargonium*. Lis-Balchin M. (Ed); Taylor & Francis: New York, 2002; Chapter 24, 262-290.

(2) Timmer, A., Gunther, J., Rucker, G., Motschall, E., Antes, G., and Kern W.V. *Pelargonium sidoides* extract for acute respiratory tract infections. *Cochrane Database Syst. Rev.* 2008; 16(3):CD006323.

(3) Kayser, O. and Kolodziej, H. Antibacterial activity of extracts and constituents of *Pelargonium sidoides* and *Pelargonium reniforme*. *Planta Med.* 1997; 63(6):508-10.

(4) Kayser, O., Kolodziej, H., and Kiderlen, AF. Immunomodulatory principles of *Pelargonium sidoides*. *Phytother. Res.* 2001; 15(12):122-6.

(5) Matthys, H. and Heger, M. EPs 7630-solution--an effective therapeutic option in acute and exacerbating bronchitis. *Phytomedicine* 2007; 14(6):65-8.

(6) Lizogub, V.G., Riley, D.S., and Heger, M. Efficacy of a *Pelargonium sidoides* preparation in patients with the common cold: a randomized, double blind, placebo-controlled clinical trial. *Explore (NY)* 2007; 3(6):573-84.

(7) Matthys, H. and Funk, P. EPs 7630 improves acute bronchitic symptoms and shortens time to remission. Results of a randomised, double-blind, placebo-controlled, multicentre trial. *Planta Med.* 2008; 74(6):686-92.

(8) Schoetz, K., Erdelmeier, C., Germer, S., and Hauer H. A detailed view on the constituents of EPs 7630. *Planta Med.* 2008; 74(6):667-74.

(9) Kayser, O., Latte, K., Kolodziej, H., and Hammerschmidt F. Composition of the essential oils of *Pelargonium sidoides* DC and *Pelargonium reniforme* Curt. *Flavour Fragrance J.* 1998; 13(3):209-12.

(10) Brendler, T. and van Wyk, B.E. A historical, scientific and commercial perspective on the medicinal use of *Pelargonium sidoides* (Geraniaceae). *J. Ethnopharmacol.* 2008; 119(3):420-33.

(11) Bladt, S, and Wagner, H. From the Zulu medicine to the European phytomedicine Umckaloabo. *Phytomedicine* 2007; 14(6):2-4.

(12) White, A.G., Davies-Coleman, M.T. and Ripley, B.S. Measuring and optimising umckalin concentration in wild-harvested and cultivated *Pelargonium sidoides* (Geraniaceae). *S. Afr. J. Bot.* 2008; 74(2):260-267.

(13) ICH Harmonised Tripartite Guideline. *Validation of Analytical Procedures: Text and Methodology Q2(R1)*. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use: Geneva, Switzerland. 2005.

تطوير طريقة بسيطة وسريعة باستخدام الكروماتوغرافيا السائلة عالية الأداء والمكشاف فوق البنفسجي لدراسة وتحليل مركب الأمكالين، كدالة نباتية، في منتج شراب السعال المحتوي على خلاصة نبات الجارديني

محمد هديب[1] ، وهف الدهاسي[2] ، أيمن خضير[3] ، رائد صلاح[4] ، حاتم الخطيب[1] ، ياسر البستنجي[1] ومحمد محمد[1]

[1] كلية الصيدلة، الجامعة الأردنية، عمان، الأردن.
[2] شركة بيوفجن، الرياض، المملكة العربية السعودية.
[3] كلية الصيدلة، جامعة العلوم التطبيقية، عمان، الأردن.
[4] شركة سنا للأبحاث الصيدلانية، عمان، الأردن.

ملخـص

تم في هذه الدراسة تطوير وإثبات أداء طريقة تحليل بسيطة وسريعة باستخدام الكروماتوغرافيا السائلة عالية الأداء والمقياس فوق البنفسجي للتحليل الكمي لمركب الأمكالين (Umckalin)، كدالة (واصمة) نباتية، في منتج شراب السعال المحتوي على خلاصة نبات الجارديني (Pelargonium). تم إجراء عملية الفصل الكروماتوغرافي باستخدام عمود فصل معكوس الطبقة الثابتة (C18) من النوع Phenomenex® (5 ميكروميتر، 25 سم x 0.5 ملم – قطر داخلي) واستخدام خليط من الأسيتونتريل وحامض الفسفوريك (درجة الحموضة 2.5) كطبقة متحركة بمعدل جريان 1 مل/دقيقة، تحت ظروف عادية (حرارة الغرفة) والقياس بواسطة الأشعة فوق البنفسجية على طول موجة 310 نانوميتر. أعطت هذه الطريقة، المستخدمة في تحديد كمية الأمكالين، منحنى معيارياً خطياً للإستجابة على مدى التراكيز من 0.334 إلى 1.667 ميكروغرام/مل وبمعامل ارتباط (r^2) مقداره 0.9996. أظهرت الدراسة كذلك أن الحدود الدنيا لتراكيز الأمكالين التي يمكن كشفها (LOD) أو قياسها كمياً (LOQ) هي بمقدار 0.0344 و0.1031 ميكروغرام/مل، على التوالي. إضافة إلى ذلك أظهرت الدراسة قيماً جيدة ومقبولة لعوامل الدقة، المتعلقة بإعادة (تكرار) التحليل، قصيرة ومتوسطة المدى، وكذلك مستويات عالية لصحة الطريقة. بساطة وثباتية أداء هذه الطريقة المطورة يجعل منها وسيلة عالية الفاعلية ومناسبة بشكل خاص لأغراض الرقابة النوعية والتحليل.

الكلمـات الدالـة: أمكالين، الجارديني (بلارجونيوم)، تحليل الكروماتوغرافيا السائلة عالية الأداء والمكشاف فوق البنفسجي، شراب السعال، منتج نباتي، دالة (واصمة) نباتية.

تاريخ استلام البحث 2009/11/22 وتاريخ قبوله للنشر 2010/3/14.