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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETERMINATION OF COLCHICINE AND RELATED COMPOUNDS

Taha M. Sarg, Meinhart H. Zenk*, Sameeh I. El-Dahmy, Afaf S. Abdel-Ghani
and Maged M. Abou-Hashem

Dept. of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt
*Institute of Pharmaceutical Biology, Munich University, Munich, Germany

ABSTRACT

An HPLC method was applied for the determination of colchicine and colchicoside in the methanolic extract of the seeds of *Colchicum autumnale* L. during different stages of seed maturity. The method is carried out isocratically on a reversed-phase column using H_2O-CH_3CN (95:5) +100 µl H_3PO_4. The detection limits were 0.10 and 0.05 µg for colchicine and colchicoside, respectively and the recoveries (99.2 ± 1.1%) were high and quantitative. Also, a highly specific and sensitive enzyme-linked immunosorbent assay (ELISA) was developed for detection and quantitative determination of colchicine, its structurally related compounds (colchicinoids) in seeds of *C. autumnale* and suspension culture of *Colchicum variegatum* L. A combined experiment (HPLC - ELISA) was performed for detection of colchicine and some other colchicinoids in *C. autumnale* seeds extract.

INTRODUCTION

Colchicine is a tropolone alkaloid which is restricted to the plants of family Liliaceae[1]. It is well known to provide effective pain relief in acute gout, arthritis[2] and in the treatment of Mediterranean fever[3]. In addition, there have been several trials for the application of colchicine in the treatment of biliary liver cirrhosis[4]. Many colchicine derivatives have been tested clinically for antitumour effect; demecolcine (N-deacetyl-N-methylcolchicine) was reported to be effective in chronic myelocytic leukemia[5]. Thus, the development of precise and sensitive methods for the detection and quantitation of colchicine and its structurally related compounds (colchicinoids) in their natural sources or in pharmaceuticals is of interest from phytochemical and medicinal point of view.

HPLC determination of colchicine has found wide application but many of these methods require special preparation procedures for the samples[6] or are concerned with the separation of colchicine rather than its quantification[7] or deals with samples in a level with narrow range[8]. Moreover, nothing has been reported concerning the quantitation of colchicoside. As an extension for the precise and sensitive determination of colchicinoids two reports dealing with enzyme immunoassay have been cited. In the first report[9], N-deacetylcolchicine was used for inducing antibodies against colchicines and the measuring range was narrow (0.05 - 5.00 ng). In the other report[10] the collected antibody that has been raised against colchicoside immunogen did not precisely recognize colchicoside. Also, the cross reactivities to colchicine photoisomers were not studied[10]. Therefore, the development of a sensitive and specific method - with a modified approach - for determination of colchicine as well as some important colchicinoids is desirable.

In this study, a combined HPLC and ELISA procedure to detect and quantify colchicine and colchicoside content in crude extracts of *Colchicum autumnale* L. seeds and suspension culture of *Colchicum variegatum* L. is presented.

EXPERIMENTAL

Apparatus:

Chromatographic analysis were carried out using HPLC *Lachrom* (Merck - Hitachi), L. 7100 pump, L. 7200 autosampler, column (RPC - 18, 250 × 4 mm), particle size 10 µm, flow rate 1 ml/min., mobile phase H_2O-CH_3CN (95:5) + 100 µl H_3PO_4 coupled with a UV photodiode array detector operating at λ max 351 nm. Sample preparation column chromabond RPC-18 propylene (1 g 7 × 1 cm) Macherey-Nagel (Düren, Germany) and precision microplate reader, Molecular Device, E max (USA).

Materials:

Seeds of *C. autumnale* were collected every week beginning in last week of April up to the end of June 1995 from plants cultivated in the experimental garden of the Institute of Pharmaceutical Biology, Munich University. The cell culture of *C.variegatum* was among the collections of cell culture laboratory of the Institute of Pharmaceutical Biology, Munich University, Munich, Germany. Authentic samples of colchicine, colchicoside, 2- and 3- dimethylcolchicine, demecolcine, α-, β-lumicolchicine and colchiceine were obtained from Indena (Italy). Bovine serum albumin (BSA), complete and incomplete forms of Freund's adjuvant, Rivanol (6,9-diaminethoxyacridine lactate), sodium periodate and horseradish peroxidase (HRP) type VI-A were obtained from Sigma (Munich, Germany). 2,2'-azinodi (3-ethyl-benzthiazoline sulfonic acid -6)ammonium salt (ABTS) was obtained from Boehringer (Mannheim, Germany).

Media:

Linsmaier and Skoog (**LS**)[11]; Murashige and Skoog (**MS**)[12] (1 L) plus thiamine HCl (0.4 mg), meso-inositol (100 mg), 2,4-dichlorophenoxy acetic acid 10^{-6}M (0.22 mg), and 1-naphthylacetic acid 10^{-6}M (0.186 mg). **4X**: Gamborg[13] (1 L) plus 2,4-dichlorophenoxy acetic acid (0.5 mg), indole-3- acetic acid (0.5 mg) and kinetin (0.2 mg). **DAX**: Gamborg (1 L) plus 2,4-dichlorophenoxy acetic acid (0.2 mg).

Methods:

Each week, 10 to 20 seeds of *C. autumnale* were collected (minimum weight of the seed was 6.5 mg and maximum weight was 21 mg). Each entire seed was digested with MeOH (1ml) at 60°C for one hr and filtered. Each extract was adjusted to 1 ml and 50 µl of the extract was injected into HPLC for quantitation. The peak areas for four repeated injections for both colchicine and colchicoside were calculated and concentrations corresponding to these peak areas were determined from the relevant calibration curves (Figs 1 and 2). Figures (3 and 4) show the relation between average seed weight (per week), colchicine and colchicoside content respectively.

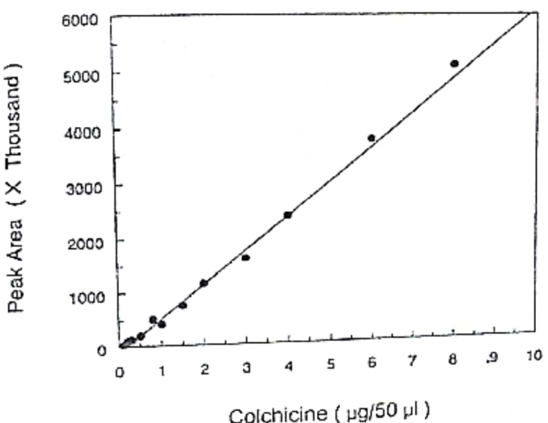

Fig. 1: HPLC standard curve for colchicine

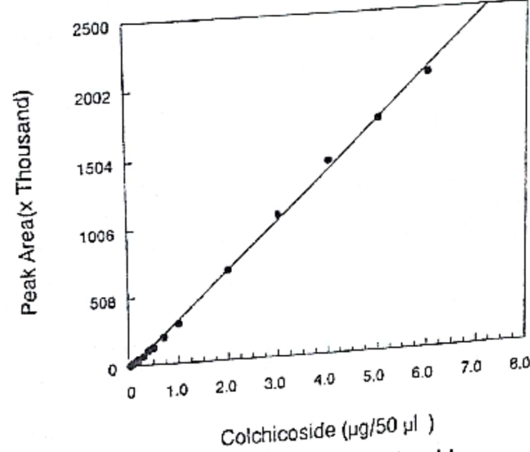

Fig. 2: HPLC standard curve for colchicoside

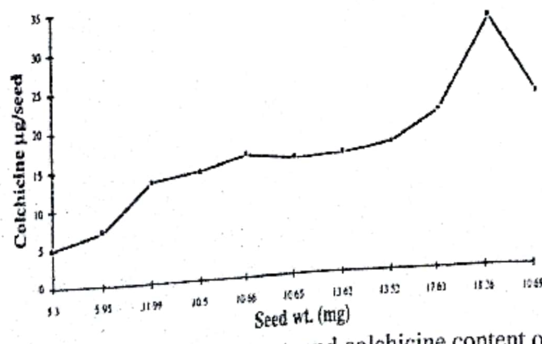

Fig. 3: Relation between wt. and colchicine content of *Colchicum autumnale* L. seeds using HPLC.

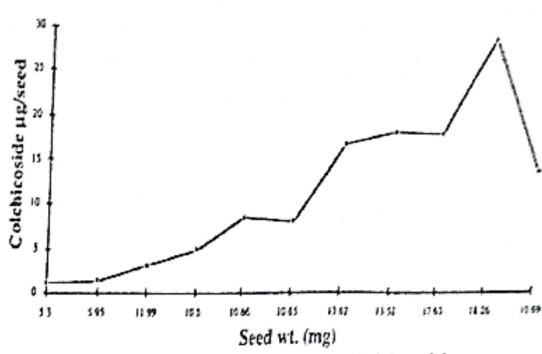

Fig. 4: Relation between wt. and colchicoside content of *Colchicum autumnale* L. seeds using HPLC.

To prove the suitability of the current colchicine HPLC method, the method was applied to isolate colchicine from a cell culture of *C. variegatum*. The cell cultures of *C.variegatum* were established on LS, 4X and DAX media.

Freshly harvested *C. variegatum* cells (68.0 g) were lyophilized to yield 5.14 g of dry cells which were extracted with methanol (50 ml) at 60°C for 1 hr. The extract was filtered and dried at 50°C under reduced pressure (1.67 g), the residue was dissolved in MeOH and subjected to PTLC (Silica gel, 0.25 mm) using $CHCl_3$-EtOAc $(C_2H_5)_2NH$ (7:4:2) as solvent system and utilizing UV detection at λ_{max} 254 and 366 nm. The band corresponding to colchicine (R_f 0.39) was scraped and extracted with hot methanol. Further purification was accomplished using HPLC; the fraction corresponding to the peak with R_t 9.89 min., was collected, concentrated and subjected to CI mass spectrometry.

I- Preparation of Colchicoside-Bovine Serum Albumin (BSA) Conjugate:

a- Preparation of colchicoside dialdehyde:

A solution of sodium periodate (109.5 mg in 1.5 ml H_2O) was added dropwise to a colchicoside solution (70 mg in 3.5 ml of 67% EtOH) over a period of 30 min., with continuous stirring at room temp. Ethylene glycol (3 drops, 50% aqueous solution) was added to neutralize excess periodate. The mixture was centrifuged for 10 min. and filtered .The filtrate was concentrated and subjected to PTLC using $CHCl_3$-EtOAc-$(C_2H_5)_2NH$ (7:4:2) as solvent system and triazin solution or antimony trichloride as spraying reagents. Colchicoside dialdehyde band (R_f 0.72) was scraped, eluted with methanol and concentrated under reduced pressure to afford 40 mg.

b- Coupling of colchicoside dialdehyde to BSA:

Colchicoside dialdehyde (20 mg) was dissolved in EtOH (5 ml, 67%), added dropwise to BSA solution (140 mg in 5 ml H_2O), adjusted at pH 9.1-9.3 using K_2CO_3 (5%, H_2O) and the solution was stirred for 90 min., at room temp. Sodium borohydride (75 mg in 5 ml H_2O) was added dropwise to the first solution, the whole mixture was stirred for three hrs. Excess sodium borohydride was neutralized by adding formic acid (1N, pH 6.5) with continuous stirring for 1 hr, the pH of the solution was adjusted to 8.5 using NH_4OH (1N). Consequently, the reaction mixture was dialysed

against deionized water for 6 days (12 × 2 L), then the conjugate was lyophilized to afford colchicoside-BSA conjugate (61 mg) which was kept at – 20°C.

c- Immunization and antiserum production:

Four albino rabbits (12- week old, weighing about 2 kg each) received intradermally (once a week for 4 consecutive weeks) 0.5 mg of the colchicoside-BSA conjugate dissolved in 250 µl 0.15 M NaCl and emulsified with an equal volume of Freund's adjuvant. The complete form of adjuvant was used in the first two weeks of initial immunization while the incomplete form was used in the following two consecutive weeks. The steps that were performed are summarized in Table 1. From the ninth week , rabbits were bled and the blood was collected, allowed to clot at room temp., for 2 hr., and centrifuged at 4000 rpm for 10 min. The antiserum was decanted, stored in aliquots in 10 ml vials and kept at –20°C.

Table 1: Immunization schedule for induction of antibody against colchicoside conjugate.

Week Nr.	Procedure
1,2	Initial immunization with colchicoside conjugate: complete Freund's adjuvant, CFA, 1:1, intradermally.
3,4	Initial immunization; colchicoside-conjugate: incomplete Freund's adjuvant, IFA, 1:1, intradermally.
5	Pause.
6	Boosting injection (1 mg conjugate in 0.5 ml) normal saline, intramuscular; in the right thigh.
7	Boosting injection (1 mg conjugate in 0.5 ml normal saline, intramuscular; in the left thigh.
8	Pause
9	Collection of blood from the marginal vein of the right inner ear.
10	Collection of blood from the marginal vein of the left, inner ear.
11	Pause

d- Isolation of the immunoglobulin fraction G (IgG) from antiserum and preparation of enzyme-labelled colchicoside was done according to Hum et al[14].

II- ELISA Procedure[10,15]:

A 96-well microtiter plate (Nunc) was coated with antibody solution (0.5 µg/well). Wells intended for determination of unspecific binding were coated with $NaHCO_3$ (200 µl, 0.01 M. pH 9). The microtiter plate was incubated overnight at 4°C. The antibody solution was discarded and the wells were washed with phosphate - buffered saline (PBS), (400 µl/well, pH 7.4). All the wells also received 200 µl each of BSA (0.5% in 0.01 M $NaHCO_3$, pH 9), incubated for 1 hr at 4°C and washed with PBS (pH 7.4). All the wells received 150 µl PBS (pH 7.4), 50 µl of either test sample (duplicate) or standard (triplicate) plus enzyme tracer (dilution 1: 8000) and mixed using a vibrating shaker for 30 sec. The plate was incubated at 37°C for 1 hr, the wells were washed twice with PBS (400 µl, pH 7.4), 200 µl of $ABTS/H_2O_2$ solution (8 mg ABTS in 20 ml phosphate-citrate buffer, pH 4.5 + 50 µl of 10-fold diluted H_2O_2 solution) was added to each well, mixed using vibrating shaker and incubated at 37°C for 90 min. The enzyme reaction was measured photometrically at λ_{max} 405 nm uising microplate reader. For calibration curve, each concentration of a serial dilutions of colchicine and colchicoside was applied in duplicates or triplicates. Standard calibration curves for cochicine and colchicoside are shown in Figures (5 and 6).Wells intended for determination of unspecific binding (**UB**) and maximal binding (**Bo**) received no sample but they received 50 µl of PBS (pH 7.4). Calculations[16,17] were made using immunoassay computation software.

% unspecific binding (UB) $= \frac{UB}{Bo - UB} \times 100$

% relative binding of the maximal binding of the enzyme tracer $\left(\frac{B}{Bo}\right) = \frac{B^* - UB}{Bo - UB} \times 100$

where B* is the sample reading.

Linear dose interpolation was obtained through the logit-log transformation: Logit [(B/Bo%)] =

$$\frac{\%B/Bo}{100 - \%B/Bo} \times 100$$

ELISA for colchicine in culture media of *C. variegatum* L.

20 g cells from LS, 4X and DAX culture media were separately extracted with MeOH (10 ml) at 60°C for 90 min. The extract was concentrated to 3 ml under reduced pressure at 45°C then chromatographed on sample preparation column [(chromabond RPC-18-propylene (1 g, 7 × 1 cm)]. Serial dilutions (1:5, 1:10, 1:50, 1:100, 1:500) were submitted for ELISA procedure.

Distribution of immunoreactive fractions in crude extract of *C. autumnale* seeds:

Five ripe entire seeds of autumnale (average weight 11 mg) were extracted with MeOH at 60°C for 90 mm. The volume was adjusted to 5 ml with MeOH and passed through sample preparation column [(chromabond, propylene (RPC-18)] the column was eluted with MeOH (3 ml), the final volume was adjusted to 5 ml 50 µl of the extract was subjected to HPLC separation using solvent system H_2O-CH_3CN (95:5) + 100 µl H_3PO_4 and UV detection at λ_{max} 351 nm. Equal fractions of 500 µl each, were collected and submitted for ELISA Identification of colchicine and colchicoside was made by comparison of R_t and UV spectra with those of standards.

RESULTS AND DISCUSSION

Colchicine and colchicoside were separated in an isocratic manner using HPLC in less than 10 min. (R_t 9.98 and 3.9 min for colchicine and colchicoside, respectively. The lower detection limit for colchicine is 0.1 µg which is twice and ten-times more sensitive than recently reported HPLC methods[6,8], while colchicoside lower detection limit is 0.05 µg. Recoveries (99.2 ± 1.1) were high and quantitative and CV was 1.8 ± 0.2. The sensitivity of the method is shown in (Figs. 1 and 2) for colchicines and colchicoside, respectively. To investigate the applicability of this method we used it to correlate the amounts of colchicine and colchicoside during different stages of maturation of *C. autumnale* L. seeds. The weekly comparison was (5:1, 4:1, 2.8:1, 1.8:1, 0.9:1 and finally 1.1:1). At the end stage, (pericarp had turned totally brown, the seeds became shrunken, reduced in weight and fragmented with great difficulty) the ratio of colchicine to colchicoside was 2:1. Definitely this contributes efficiently in tropolone biosynthetic studies[18].

The current HPLC method enabled the isolation of colchicine from a suspension culture of *C.variegatum* suspension culture. The identity of this compound was further proved by CIMS which displayed a clearly visible protonated molecular ion peak ($M^+ + 1$) at m/z 400 (100%) corresponding to molecular formula of $C_{22}H_{25}NO_6$. Further proof of identity came from spiking in HPLC and comparison of CIMS with that of an authentic standard. Comparison of HPLC with the current ELISA for colchicine was by far in favour of the latter where the working range is 0.5-10 ng/assay with a lower detection limit of 0.05 ng and recovery of 97 ±2.1. The sensitivity of ELISA for colchicine and colchicoside is demonstrated by typical curves (Fig. 5 and 6).

The specificity of the assay was determined by checking probable cross reactivity[19] with some colchicinoids. **%Cross reactivity** = [(Amount of standard at B/Bo=50%) /Amount of tested substance at B/Bo=50%)]×100.

The percentage of cross reactivity in the present and relevant recent study[10] is shown in table (2).

It is clear that the antibodies raised against the synthesized colchicoside-BSA conjugate successfully recognized colchicoside and other related tropolone alkaloids (but to a lower extent). However, prior separation of the crude extract with HPLC shows that the reacting substance is colchicine as shown in the immunochromatogram (Fig. 7). It is clear that other substances cross react with the raised antibody. This may permit the detection of the tropolon skeleton and it is possible that new compounds could be traced in such a manner. As an application of such a combination, (HPLC-ELISA), colchicine could be determined in a cell suspension culture of *C.variegatum* where DAX media was found superior in this connection (20.2 µg/L)

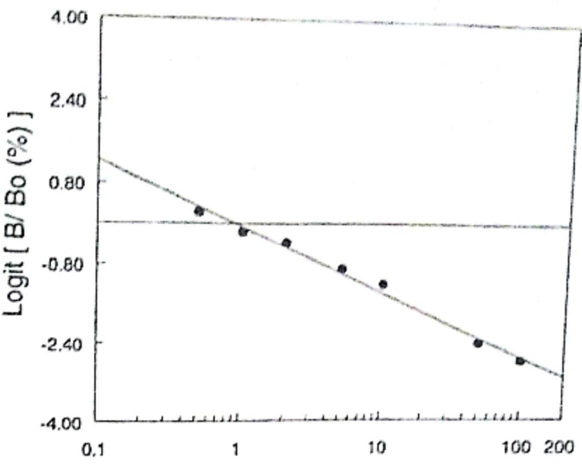

Fig. 5: Standard ELISA curve for colchicine

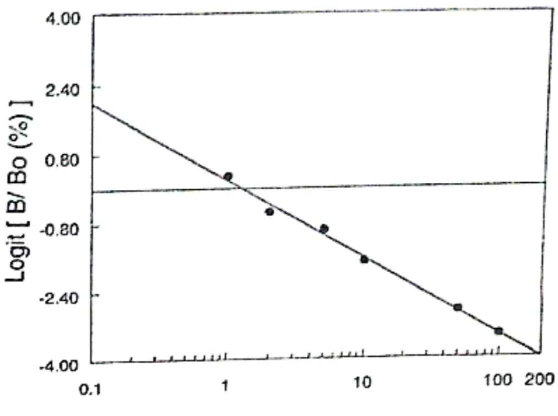

Fig. 6: Standard ELISA curve for colchicoside

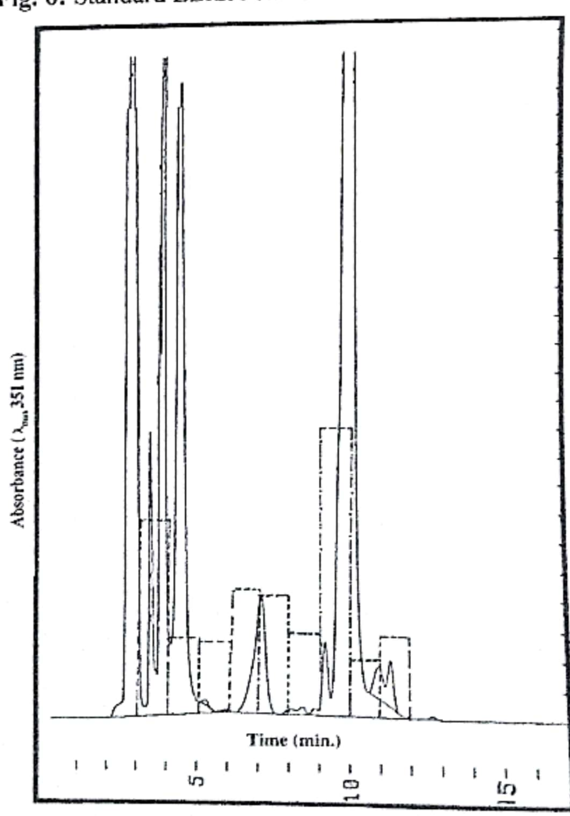

Fig. 7: Distribution of immunoreactive materials in HPLC fractions of extract of *Colchicum autumnale* seed

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Table 2: Cross reactivities of colchicine and related compounds.

Compound (M. Wt.)	Cross reactivity %		Compound (M. Wt.)	Cross reactivity %	
	present	previous [10]		present	previous [10]
Colchicine (399.4)	100	100	γ Lumicolchicine (399.2)	0	n.d
Colchicoside (547.5)	70	26	Demecolcine (371.4)	0	0
2- demethylcolchicine (R_1 = H, R_2 = CH_3)	47	26	N-deacetylcolchicine (357.4)	0	100
3- demethylcolchicine (R_1 = CH_3, R_2 = H)	14	78	Isocolchicine (399.4)	0	n.d
β-lumicolchicine (399.2)	0	n.d	Colchiceine (385.39)	0	0

n.d. = not determined

To sum up, the HPLC-ELISA system provides a highly advantageous method for detecting active tropolones as it provides a sensitive and precise determination of colchicines and other active tropolones. Both the current HPLC and ELISA methods proved to be specific and versatile methods for phytochemical investigations and biosynthetic studies .Moreover , the HPLC- ELISA system is useful to select plants of high tropolone content in plant breeding programs .

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تعيين الكولشيسين ومشتقاته بطريقتي استشراب السائل الرفيع الإنجاز والمقايسة الماصة المناعية الرابطة للإنزيم

طه مصطفي سرج و ماين هارت تسنك و سميح إبراهيم الدهمي وعفاف السيد عبد الغني وماجد محمد ماهر أبو هاشم

قسم العقاقير – كلية الصيدلة– جامعة الزقازيق – الزقازيق – مصر

* معهد البيولوجيا الصيدلية ، جامعة ميونخ – ميونخ – المانيا

يحتل الكولشسين مكانة علاجية هامة لنجاعته فى علاج النقرس والتهابات المفاصل إضافة لكونه – ومشتقاته – هدفاً فى الآونة الأخيرة للأبحاث الخاصه بالأورام السرطانية مما ضاعف من القيمة البحثية والتطبيقية لهذا القلوانى الهام .

وتتناول الدراسة الحالية طريقتين لتقدير الكولشسين وبعض مشتقاته وهما طريقة استشراب السائل الرفيع الإنجاز ذو الطور المنعكس (**HPLC-RP**) وطريقة المقايسة الماصة المناعية الرابطة للإنزيم (**ELISA**) وذلك باستخدام عينات ميليغرامية للكشف عن أجزاء من الميكروغرام من المادة الفعالة فيها .

وقد قُدّر الكولشسين وجليكوزايد الكولشسين بالطريقة الأولى فى بذور اللحلاح(*Colchicum autumnale* L.) فى مراحل متعاقبة من نمو البذور بحدود دنيا للكشف بلغت 0.05, 0.10 مكغم ونسبة استرجاع بلغت % 1.1± 99.2 للقلوانى والجليكوزايد .

كما أقيمت وطبقت الطريقة الثانيه لتقدير الكولشسين ومشتقاته فى بذور اللحلاح ومزارع الخلايا المعلقة لصنف الفاريجاتم (*Colchium variegatum* L.) وذلك ضمن مدى عمل ما بين 0.5 - 10 نانوغرام وحد أدنى للتعرف مقداره 0.05 نانوغرام فيما بلغت نسبة الاسترجاع % 2.1±97 .

هذا ويشكل الجمع بين الطريقتين وسيلة هامة للكشف عن مركبات التروبولون خاصه فى دراسات التشييد الحيوى وكذا لرصد نباتات ذات محتوى عالٍ من المادة الفعالة بما يضمن بشكل كبير نجاح نظم التهجين للنباتات ذات الأهميه العلاجيه .