List of chemicals/reagents

S. No. Chemicals/Reagents	Purity/Grade	Manufacturer
1. Hypericin	98%	Enzo Life Sciences AG, Switzerland
2. Khellin	98%	Fluka, USA
3. Podophyllotoxin	98%	Fluka, USA
4. Etoposide	98%	Sigma Aldrich, USA
5. Strychnine	98%	Fluka, USA
6. Brucine	98%	Sigma Aldrich, USA
7. Vincristine	98%	Vinkem Labs Pvt. Ltd, India
7. Vinblastine	98%	Vinkem Labs Pvt. Ltd, India
8. Acetonitrile	(HPLC grade)	Merck Specialities Pvt. Ltd.
9. Methanol	(HPLC grade)	Merck Specialities Pvt. Ltd.
10. Ammonium acetate	(AR grade)	Merck Specialities Pvt. Ltd.
11. Water	(HPLC grade)	Millipore
12. Toluene	(AR grade)	S. D. fine-chem. Ltd.
13. Diethylamine	(AR grade)	Merck Specialities Pvt. Ltd.
14. Dichloromethane	(AR grade)	Merck Specialities Pvt. Ltd.
15. Formic acid	(AR grade)	S. D. fine-chem Ltd.
16. Chloroform	(AR grade)	S. D. fine-chem Ltd.
17. Methanol	(AR grade)	Merck Specialities Pvt. Ltd.

List of instruments

S. No.	Instruments	Manufacturer
1	HPLC (Quaternary system)	Shimadzu corporation, Japan
2	HPTLC	Camag, Switzerland
3	UV-VIS Spectrophotometer	Shimadzu corporation, Japan
4	Electronic Microbalance	Mettler Toledo, Switzerland

4.1. Development of analytical method for HPLC of anticancer drug hypericin

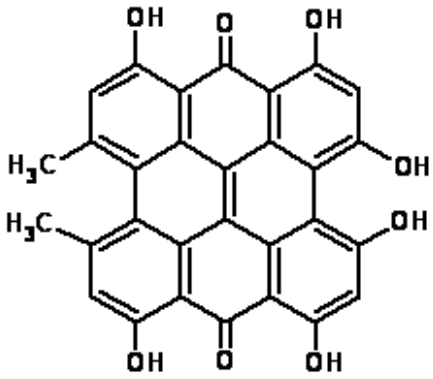


Figure 2. Chemical structure of hypericin

4.1.1. Preparation of standard solutions

Standard solution of hypericin was prepared by dissolving 1.0 mg of hypericin in 10 mL of methanol (100 µg mL^{-1}). From this stock solution, further dilutions like 10, 20, 40, 60, 80 (µg mL^{-1}) were made to obtain calibration curve of hypericin.

4.1.2. Extraction of hypericin from plant

Hypericin was extracted from aerial parts of *Hypericum perforatum* L. plant. The extraction of hypericin was performed by weighing 50 gm of dried powdered drug from plant and extracted it with ethanol (80%, v/v; 250 mL × 3) in a blender with stirring. The hydro-alcoholic extract was defatted by liquid liquid extraction with hexane (until colorless) and concentrated to a final amount of 14.65 gm dried drug. This sample after suitable dilution with methanol (1.0 mg mL^{-1}) was filtered through a 0.2 µm non sterile regenerated cellulose membrane (Sartorius AG, Germany).

4.1.3. HPLC conditions

All samples including hypericin standards and *H. perforatum* extracts were analyzed separately on an HPLC system with standard methods. The stock solutions (100 µg mL^{-1}) of hypericin standards and extracts used in the analysis were prepared in methanol. The HPLC system (Shimadzu Corporation, Japan) consisted of a binary pump (model LC -10AT VP), a UV-VIS detector (model SPD-10AVP), a rheodyne injector (model 77251) equipped with CLASS-VP software (Version 5.032) was used to separate hypericin. A reverse phase C-18 Column (Phenomenex, USA, 5 µm, 250 × 4.6 mm) attached to a guard column (Shimadzu Corporation Japan, 5 µm, 10 × 4.0 mm) was used to separate hypericin. The mobile phase used consisted of acetonitrile, methanol, 10 mM ammonium acetate (pH 5.0) in the ratio of 54: 36: 10, v/v/v). The flow rate of the HPLC system was set at 1.0 mL min^{-1} and the run time was 16 min per sample. The applied volume of the sample was 20 µL and hypericin was detected at 590 nm. The validation parameters such as linearity, LOD, LOQ, precision, recovery and robustness were calculated as per the ICH guidelines (International Conference on Harmonization, Nov. 2005). The quantitative analysis was carried out by comparing the retention time and peak area of the standards and that of the sample extracts.

4.1.4. Method validation

4.1.4.1. Linearity

Five point calibration curve was constructed by plotting peak area against concentration. Linearity was evaluated by applying each concentration (10, 20, 40, 60, 80 µg mL^{-1}) of hypericin in triplicates per sample and five such samples were evaluated (n = 3 × 5).

4.1.4.2. Precision

Precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. Repeatability was determined by six replicate applications and six

times measurement of a standard solution at the analytical concentration of 30, 40 and 60 µg mL^{-1} of hypericin. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of percent relative standard deviation (% RSD). Precision was obtained from % RSD value by repeating the assay six times on the same day for intra-day precision. Intermediate precision was assessed by the assay of three, six sample sets on different days (inter-day precision) and on different system (inter-system precision). The intra-day, inter-day and inter-system variations for determination of hypericin were carried out at three different concentration levels 30, 40 and 60 µg mL^{-1}.

4.1.4.3. Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like acetonitrile: methanol: ammonium acetate (pH 5.0) (53.8: 36.2: 10 and 54.2: 35.8: 10, v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied in the range of ± 0.2%. Robustness of the method was done at three different concentration levels 30, 40 and 60 µg mL^{-1}. The wavelengths of the UV-Vis detector were also changed (590 ± 2 nm) and % RSD were determined and found to be less than 2 %.

4.1.4.4. Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank solution (methanol) was spotted six times following the same method as explained above and the signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting known concentrations of reference solution until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

4.1.4.5. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The peak for hypericin in sample was confirmed by comparing Rt and the presence of hypericin was also confirmed by comparing UV spectra of sample with that of standard.

4.1.4.6. Recovery

The pre-analyzed samples were spiked with 0, 50, 100 and 150% of the standard solution and the mixtures were re-analyzed by the proposed method. The experiment was conducted six times. This was done to check the recovery of the drug at different levels in the formulations. Recovery study was carried out for the powdered sample of *H. perforatum.*

4.2. Development of analytical method for HPLC of antispasmodic drug khellin

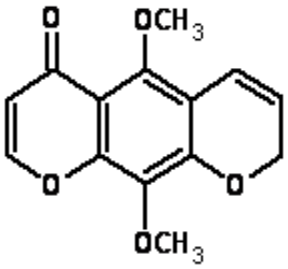


Figure 3. Chemical structure of khellin

4.2.1. Preparation of standard solutions

Standard solution of khellin was prepared by dissolving 10 mg of khellin in 10 ml. of methanol (1000 µg mL^{-1}) From this stock solution, further dilutions were made to obtain calibration graph of khellin.

4.2.2. Preparation of sample solutions

The sample was prepared by diluting 200 µL of liquid herbal extract of khellin in 10 mL of methanol (20 µL mL^{-1}) and was filtered through a 0.2 µm non sterile regenerated cellulose membrane (Sartorius AG, Germany).

4.2.3. HPLC conditions

All samples including khellin standards and *Ammi visanaga* extracts were analyzed separately on an HPLC system with standard methods. The stock solutions (1000 µg mL^{-1}) of khellin standards and extracts used in the analysis were prepared in methanol. The HPLC system (Shimadzu Corporation, Japan) used consisted of a binary pump (model LC-10AT VP), a UV-VIS detector (model SPD-10AVP), a rheodyne injector (model 77251) equipped with CLASS-VP software (Version 5.032). A reverse phase C-18 column (5.0 µm particle, 250 × 4.6 mm) from Lichrocart, Germany was used to separate khellin. The mobile phase used consisted of methanol: water in

the ratio of (75: 25, v/v). The flow rate of the HPLC system was set at 1.0 mL min^{-1} and the run time was 10 min per sample. The applied volume of the sample was 20 µL and khellin was detected at 247 nm. The validation parameters such as linearity, LOD, LOQ, precision, recovery and robustness were calculated as per the ICH guidelines (International Conference on Harmonization, Nov. 2005). The quantitative analysis was carried out by comparing the retention time and peak area of the standards and that of the sample extracts.

4.2.4. Method Validation

4.2.4.1. Linearity

Linearity was found between concentration range of 10-80 µg mL^{-1} for khellin with r2 ± SD = 0.999 ± 0.001. Linearity was evaluated by applying each concentration (10-80 µg mL^{-1}) for khellin in triplicates per sample and five such samples were evaluated (n = 3 × 5).

4.2.4.2. Precision

Precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. System repeatability was determined by six replicate applications and six times measurement of a standard solution at the analytical concentration of 20, 40 and 50 µg $mL^{-1}$of khellin. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of relative standard deviation (RSD). Method repeatability was obtained from RSD value by repeating the assay six times on the same day for intra-day precision. Intermediate precision was assessed by the assay of three, six sample sets on different days (inter-day precision) and on different system (inter-system precision). The intra-day, inter-day and inter-system variations for determination of khellin were carried out at three different concentration levels 20, 40 and 50 µg mL^{-1}.

4.2.4.3. Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phase having different compositions like methanol: water (75: 25, v/v) was tried Robustness of the method was carried out at three different concentration levels 20, 40 and 50 µg mL^{-1}. The detection wavelength was also changed (± 2 nm) and % RSD was determined in result and chromatograms were analysed. The amount of mobile phase was varied in the range of ± 0.2%.

4.2.4.4. Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank solution (methanol) was spotted six times following the same method as explained above. The signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting known concentrations of reference solution until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

4.2.4.5. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The peak for khellin in the sample was confirmed by comparing Rt and UV spectra of peak with that of standard. The peak purity (90%) of khellin was assessed by comparing the spectra at three different levels i.e. peak start, peak apex and peak end positions of the spot. Purity of sample peak corresponding to khellin was determined by taking the spectra and by comparing it with that of standard.

4.2.4.6. Recovery

The pre-analyzed samples were spiked with 0, 50, 100 and 150% of the standard solution and the mixtures were re-analyzed by the proposed method. The experiment was performed six times. This was done to check the recovery of the drug at different levels in the formulations. Recovery study was carried out for the liquid sample of the khellin extract.

4.3. Development of simultaneous analytical method for HPTLC of anticancer drugs vincristine and vinblastine

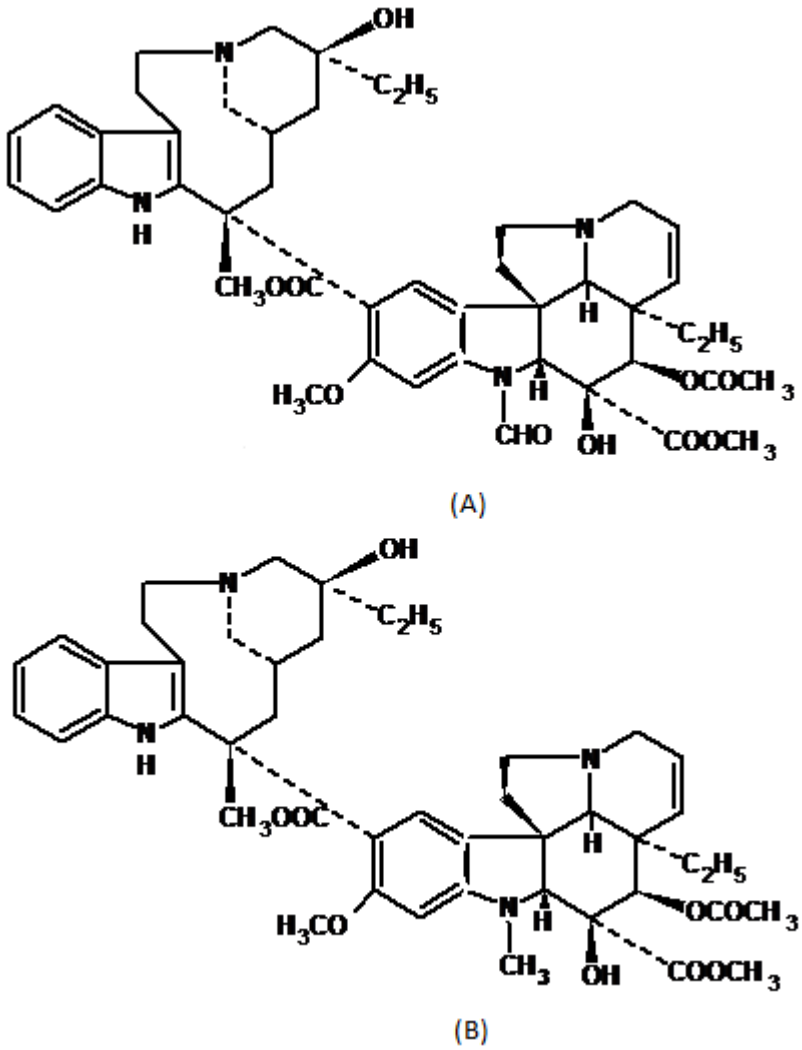


Figure 4. Structures of vincristine (A), vinblastine (B)

4.3.1. Preparation of standard solutions

Standard solutions of vincristine and vinblastine were prepared by dissolving 10mg each of vincristine and vinblastine in 10ml.of methanol (1000µg/ml). This stock solution was used to make calibration curves of vincristine and vinblastine.

4.3.2. Extraction of vincristine and vinblastine from plant

Weighed 50 gm of *Catharanthus Roseus* leaves and boiled it for two hours on an electric water bath. Powdered the leaves and then mixed it with sufficient quantity of alcoholic KOH and dried the powder in oven at 100°C. An accurately weighed quantity (2.0 g) of leaves were sonicated for 20 minutes in 4.0 mL of methanol separately. The solutions were filtered and collected in vials. Extracted the drug with 150 mL methanol in soxhlet apparatus for six hours. Methanol extract was separated and shaken with successive three portions of 5.0 mL dilute sulphuric acid. Combined the acid extract and then filtered. Added excess of ammonia to the acid extract to precipitate the alkaloids. Filtered and dried precipitate was weighed. The precipitate was then dissolved in methanol (200 mg mL^{-1}).

4.3.3. HPTLC conditions

All samples including vincristine and vinblastine standards and *Vinca rosea* extracts were analyzed separately on an HPTLC system with standard methods. The stock solutions (1000 µg mL^{-1}) of vincristine and vinblastine standards and extracts used in the analysis were prepared in methanol. Sample solutions were applied onto the plates with semi-automatic TLC sampler Linomat V (Camag, Muttenz, Switzerland) and were controlled by WinCATS software 1.4.4. Plates were developed in 20 x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). A TLC scanner III was used for scanning the TLC plates. Pre-coated silica gel aluminium plates 60F254 (E. Merck, Darmstadt, Germany) with thickness 0.2 mm thickness were used for all determinations. The plates were pre-washed with methanol and activated at 60°C for five minutes prior to chromatography. Six different aliquots (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 4.0 µL) of standard solution were applied on 20 x 10 cm TLC plates for the preparation of calibration curve. A constant application rate of 150 nL s^{-1} was employed with a band width of 7.0 mm.The slit dimension was kept at 6.0 mm x 0.45 mm and scanning speed of 20 mm/s was employed.

Twenty mL of mobile phase consisted of toluene-methanol-diethylamine (8.75: 0.75: 0. 5, v/v/v) was used per plate. The optimized chamber saturation time for mobile phase was 15 min at room temperature (25 ± 2°C) at relative humidity of 60 % ± 5 RH. The plates were developed and scanned within 10 min using desitometric scanner III in the remission mode at 307 nm for vincristine and 225 nm for vinblastine, respectively. The source of radiation was deuterium lamp emitting a continuous radiation between 200-400 nm. Evaluation was done by measuring peak areas with linear regression. The validation parameters such as linearity, LOD, LOQ, precision, recovery and robustness were calculated as per ICH guidelines (International Conference on Harmonization, Nov. 2005). The quantitative analysis was carried out by comparing the retention time and peak area of the standards and that of the sample extracts.

4.3.4. Method Validation

4.3.4.1. Linearity

Six point calibration curve was constructed by plotting peak area against concentrations. Linearity was evaluated by applying each concentration (200, 400, 1600, 3200, 4000 ng $spot^{-1}$) of vincristine and vinblastine in triplicates per sample and five such samples were evaluated (n = 3 × 6).

4.3.4.2. Precision

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. Repeatability was determined by six replicate applications and six times measurement of a standard solution at the analytical concentration of 400, 1600 and 3200 ng $spot^{-1}$ of vincristine and vinblastine. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of relative standard deviation (%RSD). Precision was obtained from %RSD value by repeating the assay six times on the same

day for intra-day precision. Intermediate precision was assessed by the assay of three, six sample sets on different days (inter-day precision) and on different systems (inter-system precision). The intra-day, inter-day and inter-system variations for determination of vincristine and vinblastine were carried out at three different concentration levels 400, 1600 and 3200 ng $spot^{-1}$.

4.3.4.3. Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like toluene-methanol-diethylamine (8.75: 0.75: 0.5, v/v/v) were tried and chromatograms were run. The amount of mobile phase was varied in the range of ±5%. The plates were pre-washed by methanol and activated at 60 ± 5°C for 5, 10, 12 min prior to chromatography. Robustness of the method was done at three different concentration levels 400, 1600 and 3200 ng $spot^{-1}$. Amount of mobile phase was varied and plates were developed in 8, 10 and 12 mL mobile phase. Time from spotting to chromatography and chromatography to scanning were also varied and RSD were determined and found to be less than 2 %.

4.3.4.4. Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank solution (methanol) was spotted six times following the same method as explained above. The signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting known concentrations of reference solution until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

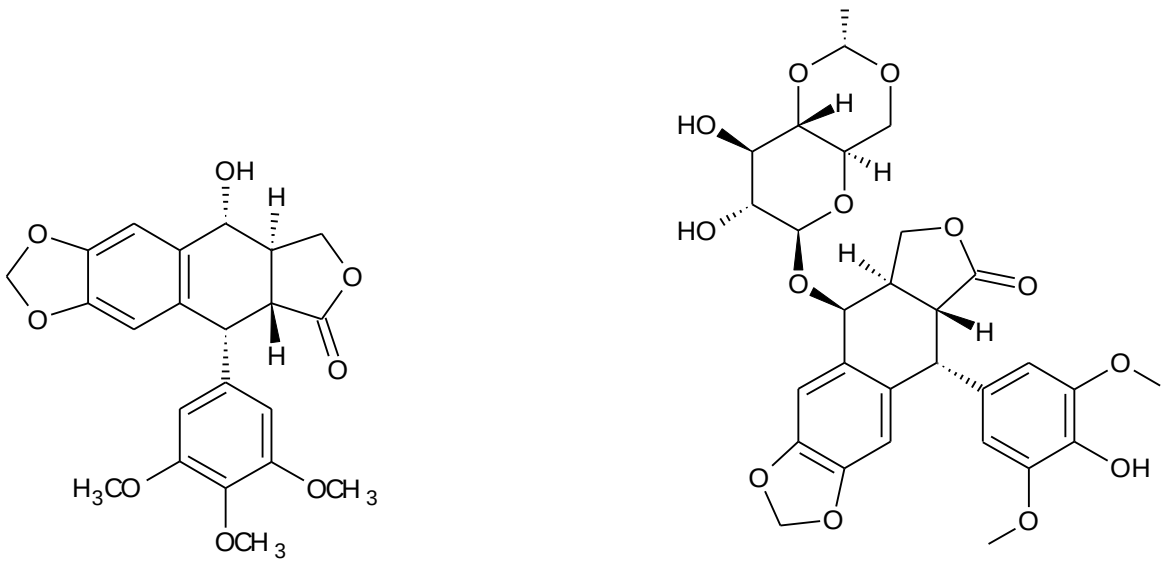
4.3.4.5. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spots for vincristine and vinblastine in sample were confirmed by comparing Rf and spectra of spot with that of standard. The peak purity (90%) of vincristine and vinblastine was assessed by comparing the spectra at three different levels i.e. peak start, peak apex and peak end positions of the spot. Purity of sample spot corresponding to vincristine and vinblastine was determined by taking the spectra and by comparing it with that of standard.

4.3.4.6. Recovery

The pre-analyzed samples were spiked with 50, 100 and 150% of the standard solution and the mixtures were re-analyzed by the proposed method. The experiment was conducted six times. This was done to check the recovery of the drug at different levels in the formulations. Recovery study was carried out for the powder sample of Catharanthus Roseus powder from a Delhi market.

4.4. Development of analytical method for HPTLC of anticancer drugs podophyllotoxin and etoposide



(a) (b)

Figure 5. Structures of (a) Podophyllotoxin, (b) Etoposide

4.4.1. Preparation of standard solutions

A standard solution containing podophyllotoxin was prepared by dissolving 1.5 mg in 3 mL of methanol (500 µg mL^{-1}) whereas a standard solution of etoposide was prepared by dissolving 2.5 mg in 2.5 mL of methanol (1000 µg mL^{-1}). This stock solutions were used to make calibration curves of podophyllotoxin and etoposide.

4.4.2. Extraction of podophyllotoxin from plant

The dried and pulverized roots of *Podophyllum hexandrum* roots (60 g) were extracted with MeOH (200 mL) in a soxhlet over water bath for 6 h. The extract was filtered and solvent was removed in Rotavapor at 50°C. The concentrated extract was redissolved in HPLC grade and volume was adjusted to 1.0 mL each. The extracted portions were combined and concentrated by

evaporation under reduced pressure to give a crude extract (9.24 g), which was dissolved in MeOH (100 mL)

4.4.3. Preparation of formulation sample

The formulation samples were made by diluting marketed formulation Etosid (Cipla make) containing etoposide with methanol. This was done by taking 0.2 mL of this formulation (100 mg $5mL^{-1}$) in 2.0 mL of methanol.

4.4.4. HPTLC conditions

All samples including podophyllotoxin and etoposide standards and *Podophyllum hexandrum* extracts were analyzed separately on an HPTLC system with standard methods. The stock solutions (1000 µg mL^{-1}) of podophyllotoxin and etoposide standards and extracts used in the analysis were prepared in methanol. Sample solutions were applied with semiautomatic TLC sampler Linomat V (Camag, Muttenz, Switzerland) controlled by WinCATS software 1.4.4. The plates were developed in 20 x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). A TLC scanner III was used for scanning the TLC plates. Pre-coated silica gel aluminium plates 60F254 (E. Merck, Darmstadt, Germany) with thickness 0.2 mm were used for all determinations. The plates were pre-washed with methanol and activated at 60°C for five minutes prior to chromatography. Five different volumes (0.3, 0.6, 1.2, 2.4, 4.8 µL) of standard solution of podophyllotoxin were applied on 20 x 10 cm TLC plate for the preparation of calibration curve of podophyllotoxin. Similarly, five different volumes (0.2, 0.4, 0.8, 1.0, 2.0 µL) of standard solution of etoposide were applied on 20 x 10 cm TLC plate for the preparation of calibration curve of etoposide. A constant application rate of 150 nL s^{-1} was employed for both podophyllotoxin and etoposide with a band width of 6.0 mm and 5.0 mm for podophyllotoxin and etoposide, respectively. The scanning speed was employed at 20 mm s^{-1} for both

podophyllotoxin and etoposide and the slit dimension was kept at 5.0 mm x 0.45 mm and 4.0 mm x 0.45 for podophyllotoxin and etoposide, respectively. Twenty mL of mobile phase consisted of dichloromethane-methanol-formic acid (9.5: 0.5: 0. 5, v/v/v) was used per plate. The optimized chamber saturation time for mobile phase was 15 min at room temperature (25 ± 2 º C) at relative humidity of 60 ± 5 % RH. The plates were developed and scanned within 10 min using desitometric scanner III in the absorbance mode at 292 nm for both podophyllotoxin and etoposide. The source of radiation was deuterium lamp emitting a continuous radiation between 200-400 nm. The data obtained were analysed by WinCATS software to get linear regression equation. The validation parameters such as linearity, LOD, LOQ, precision, recovery and robustness were calculated as per the ICH guidelines (International Conference on Harmonization, Nov. 2005). The quantitative analysis was carried out by comparing the retention time and peak area of the standards and that of the sample extracts.

4.4.5. Method Validation

4.4.5.1. Linearity

A five point calibration curve was constructed by plotting peak area against concentrations. Linearity was evaluated by applying different concentrations 150 to 2400 ng $spot^{-1}$ for podophyllotoxin and 200 to 2000 ng $spot^{-1}$ for etoposide in triplicates per sample and five such samples were evaluated (n = 3 × 5).

4.4.5.2. Precision

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. System repeatability was determined in six replicates of a standard solution at three concentration levels of 400, 800 and 1600 ng/spot and 200, 400, 800 ng/spot for podophyllotoxin and etoposide, respectively. The results of repeatability were expressed in terms

of relative standard deviation (%RSD). Intra-day precision was done by repeating the same assay six times on the same day. Intermediate precision was also assessed by the assay of three; six standard solutions were sets on different days (inter-day precision) and on different system (Inter-system precision). The intra-day, inter-day and inter-system variations for determination of podophyllotoxin and etoposide were carried out at three different concentration levels of 400, 800 and 1600 ng spot^{-1} and 200, 400, 800 ng spot^{-1} respectively.

4.4.5.3. Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like dichloromethane: methanol: formic acid (9.7: 0.3: 0.5, v/v/v) and (9.3: 0.7: 0.5, v/v/v) were tried and chromatograms were run. The volume of mobile phase was varied in the range of ± 5%. The plates were pre-washed by methanol and activated at 60 ± 5°C for 5, 10 and 12 min prior to chromatography. Robustness of the method was done at three different concentration levels 400, 800 and 1600 ng spot^{-1} and 200, 400, 800 ng spot $^{-1}$ for podophyllotoxin and etoposide, respectively. Plates were developed in varied volume of mobile phase 8, 10 and 12 mL. Time from spotting to chromatography and chromatography to scanning were also varied and %RSD was determined and found to be less than 2 %.

4.4.5.4. Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank solution (methanol) was spotted six times following the same method as explained above. The signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting known concentrations of reference solution until the average

responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

4.4.5.5. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spots for podophyllotoxin and etoposide in sample were confirmed by comparing Rf and spectra of spot with that of standard. The peak purity of podophyllotoxin and etoposide was assessed by comparing the spectra at three different levels i.e. peak start, peak apex and peak end positions of the spot. Purity of sample spot corresponding to podophyllotoxin and etoposide was determined by taking the spectra and by comparing it with that of standard.

4.4.5.6. Recovery

The pre-analyzed samples were spiked with 50, 100 and 150% of the standard solution and the mixtures were reanalyzed by the proposed method. The experiment was conducted six times. This was done to check the recovery of the drug at different levels in the crude drug.

4.5. Development of simultaneous analytical method for HPTLC of anticancer drugs strychnine and brucine

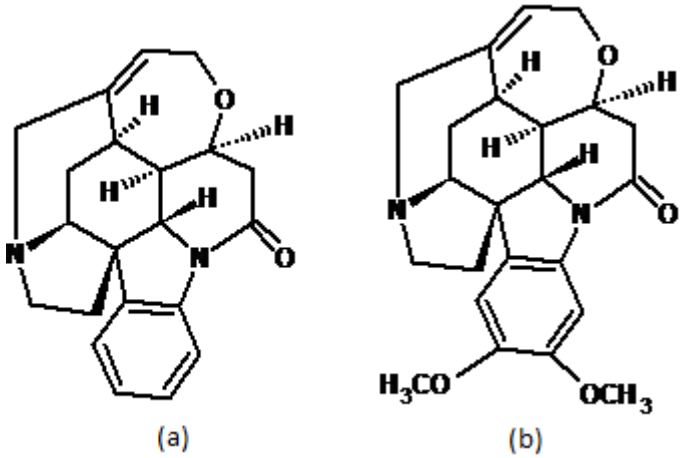


Figure 6. Structures of strychnine (a), brucine (b)

4.5.1. Preparation of standard solutions

A standard solution containing strychnine and brucine was prepared by dissolving five mg each in 10 mL of methanol (500 µg mL^{-1}).This stock solution was used to make calibration curves of strychnine and brucine.

4.5.2. Extraction of strychnine and brucine from plant

Weighed 50 g of nux-vomica seeds and boiled it for two hours on water bath. The seeds were powdered and mixed with sufficient quantity of alcoholic KOH and dried in an oven at 100□ C. Accurately weighed quantity (10 g) of seed powder was extracted with 200 mL of $CHCL_3$ in soxhlet apparatus and concentrated to 50 mL. Chloroform extract was shaken with successive three portions of dilute sulphuric acid (50 mL each). Combined the acid extract and filtered, added excess amount of ammonia to the acid extract to precipitate the alkaloids. The alkaline mixture was successively extracted with chloroform thrice (100 mL each) to ensure complete extraction. The chloroform extract was passed over sodium sulphate and evaporated to dryness

on water bath. The residue obtained was reconstituted in 10 mL of methanol and used for quantification.

4.5.3. HPTLC conditions

All samples including strychnine and brucine standards and *nux-vomica* extracts were analyzed separately on an HPTLC system with standard methods. The stock solutions (1000 µg mL^{-1}) of strychnine and brucine standards and extracts used in the analysis were prepared in methanol. Sample solutions were applied with semiautomatic TLC sampler Linomat V (Camag, Muttenz, Switzerland) controlled by WinCATS software 1.4.4. The plates were developed in 20 x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). A TLC scanner III was used for scanning the TLC plates. Pre-coated silica gel aluminium plates 60F254 (E. Merck, Darmstadt, Germany) with thickness 0.2 mm were used for all determinations. The plates were pre-washed with methanol and activated at 60°C for 5 minutes prior to chromatography. Six different volumes (0.1, 0.2, 0.4, 0.8, 1.0, 2.0 µL) of mixed standard solution (strychnine and brucine) were applied on 20 x 10 cm TLC plate for the preparation of calibration curves of strychnine and brucine. A constant application rate of 150 nL s^{-1} was employed with a band width of 7.0 mm. The slit dimension was kept at 6.0 mm x 0.45 mm and scanning speed of 20 mm s^{-1} was employed. Twenty mL of mobile phase consisted of chloroform: methanol: formic acid (8.5: 1.5: 0.4, v/v/v) was used per plate. The optimized chamber saturation time for mobile phase was 15 min at room temperature (25 ± 2 º C) at relative humidity of 60 ± 5 % RH. The plates were developed and scanned within 10 min using desitometric scanner III in the absorbance mode at 259 nm and 306 nm for strychnine and brucine, respectively. The source of radiation was deuterium lamp emitting a continuous radiation between 200-400 nm. The data obtained were analysed by WinCATS software to get linear regression equation. The validation parameters

such as linearity, LOD, LOQ, precision, recovery and robustness were calculated as per the ICH guidelines (International Conference on Harmonization, Nov. 2005). The quantitative analysis was carried out by comparing the retention time and peak area of the standards and that of the sample extracts.

4.5.4. Method Validation

4.5.4.1. Linearity

A six point calibration curve was constructed by plotting peak area against concentrations. Linearity was evaluated by applying each concentration (50 to 1000 ng spot^{-1}) for strychnine and brucine in triplicates per sample and six such samples were evaluated (n = 3 × 6).

4.5.4.2. Precision

The precision of a method is the extent to which the individual test results of multiple injections of a series of standards agree. System repeatability was determined in six replicates of a standard solution at three concentration levels of 100, 200 and 400 ng spot $^{-1}$ of strychnine and brucine, respectively. The results of repeatability were expressed in terms of relative standard deviation (%RSD). Intra-day precision was done by repeating the same assay six times on the same day. Intermediate precision was also assessed by the assay of three; six standard solutions were sets on different days (inter-day precision) and on different system (Inter-system precision). The intra-day, inter-day and inter-system variations for determination of strychnine and brucine were carried out at three different concentration levels 100, 200 and 400 ng spot^{-1}.

4.5.4.3. Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions like chloroform: methanol: formic acid (8.7: 1.3: 0.4, v/v/v) and (8.3: 1.7: 0.4, v/v/v) were tried and chromatograms were run. The

volume of mobile phase was varied in the range of ± 5%. The plates were pre-washed by methanol and activated at 60 ± 5°C for 5, 10 and 12 min prior to chromatography. Robustness of the method was done at three different concentration levels 400, 600 and 800 ng $spot^{-1}$. Plates were developed in varied volume of mobile phase 8, 10 and 12 mL. Time from spotting to chromatography and chromatography to scanning were also varied and %RSD was determined and found to be less than 2 %.

4.5.4.4. Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank solution (methanol) was spotted six times following the same method as explained above. The signal to noise ratio was determined. LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting known concentrations of reference solution until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

4.5.4.5. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spots for strychnine and brucine in sample were confirmed by comparing Rf and spectra of spot with that of standard. The peak purity of strychnine and brucine was assessed by comparing the spectra at three different levels i.e. peak start, peak apex and peak end positions of the spot. Purity of sample spot corresponding to strychnine and brucine was determined by taking the spectra and by comparing it with that of standard.

4.5.4.6. Recovery

The pre-analyzed samples were spiked with 50, 100 and 150% of the standard solution and the mixtures were reanalyzed by the proposed method. The experiment was conducted six times. This was done to check the recovery of the drug at different levels in the crude drug.