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Optimization of Extraction Conditions and HPTLC - UV Method for Determination of Quinine in Different Extracts of C*inchona species* Bark

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Abstract: A simple, precise and accurate high-performance thin-layer chromatographic method has been established for quantitative determination of quinine. Conditions were also optimized for best possible extraction of quinine via varying concentrations of diethyl amine in different solvents (*n*-hexane, chloroform, ethyl acetate and methanol) for maximum recovery of quinine. Methanol modified with 20 % DEA found to be best for highest possible recovery of target analyte quinine. Chromatographic separation of quinine was performed on silica gel 60 F_{254} HPTLC plates with ethyl acetate : diethyl amine in the proportion 88 : 12 (*v/v*), as mobile phase. The determination was carried out using the densitometric absorbance mode at 236 nm. Quinine response was found to be linear over the range 4–24 µg spot^{-1}. The HPTLC method was evaluated in terms of specificity, precision, reproducibility, LOD – LOQ and robustness. Beside these parameters, number of theoretical plates and flow constant were also included as a part of validation.

Keywords: *Cinchona spp.*; Rubiaceae; bark; quinine; HPTLC determination.

1. Introduction

Herbal drugs have been in use by different civilizations in different parts of the world for centuries to fight a large number of diseases. Many of these are in common use even today. Cinchona is a genus of family Rubiaceae, native to tropical South America. They are large shrubs or small trees

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growing to 5-15 meters tall with evergreen foliage. The bark of cinchona produces several alkaloids, the most important alkaloid, quinine (Figure 1), has certain febrifuge properties. Quinine was used in the battle against malaria since the 1620's. The bark of wild species may yield a quinine content of as high as 7%, whereas cultivated crops yield contents up to 15% [l]. Off many species of cinchona, only four species have economic value for the production of quinine: *C. calisaya*, *C. legeriana*, *C. officianalis* and *C. succirubra*. In the mid 1800's, Clements Markham, J. C. Hasskarl, and Charles Ledger collected young plants and seeds for crops. Hasskarl and Markham's early attempts to grow cinchonas in India and Java were not successful due to difficulties with transporting young trees, low quinine yield. Clements Markham with the help of Richard Spruce collected trees and seeds of *C. succirrubra* and transported them to India successfully in 1860. South America, British India and Java traded varieties of cinchonas to achieve more variation. Java quickly became the largest producer accounting for 95% of the world's commercial supply. Today other better antimalarial drugs exist. Still, in addition to its medicinal use quinine is used as a tonic, an antiseptic, and lotions [2]. Quinine is chiefly used in the teatment of falciparum malaria resistant to other antimalarials (quinacrine, chloroquine, and primaquine). Quinine is preferred where the disease has become highly resistant to other antimalarial drugs. In addition to its antimalarial activity, quinine shows antibacterial, antipyretic, mild oxytocic, local anesthetic, cardiovascular stimulant and analgesic properties, and it decreases the excitability of motor endplate. Quinine is used to prevent cardiac arrythmias and is used in tonic beverages, which are mixed with alcohols for bitter taste. Quinine is one of the most useful alkaloids for pharmaceutical purposes [3].

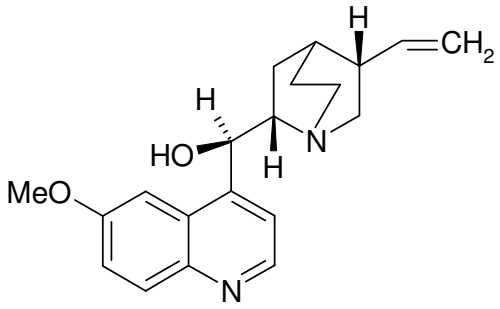

Figure 1. Structure of Quinine ($C_{20}H_{24}N_2O_2$; MW = 324)

A lot of work have been done for the determination of quinine in pharmaceuticals and biological fluids by HPLC / RP-HPLC [4-6] but a few reports are available for the determination of it in crude bark extracts as well as in marketed formulations [7, 8]. Mroczek and Glowniak (2000) [8] performed separation of quinine on to TLC and HPTLC from extract of cinchona cortex and pharmaceutical preparations using quaternary mobile phase. International pharmacopoeia reports a ternary mobile phase for quinine separation. Here, we developed a method for extraction as well as quantitative determination of quinine from bark of *Cinchona species* using binary mobile phase. The method is not only fast but also provides data for large scale processing of quinine from its raw material. This method will be helpful in quality control and quantitative studies for various industries as well as in the search for development of high yielding plant varieties either by plant breeding or biotechnological studies.

2. Materials and Methods

2.1. Plant Material

Cinchona spp. bark was purchased from local market. Bark was pulverized to a fine powder of 14 mesh in a mechanical blender. This fine powder was utilized for experimental purpose.

2.2. Chemicals

All solvents and diethyl amine (DEA) used in this study were of analytical grade. Reference standard of quinine was purchased from E. Merck, Darmstadt, Germany (Merck code no. 8.02304.0025).

2.3. Apparatus

Scanning of HPTLC plates were performed on a camag's computerized densitometer scanner 3 controlled by *winCATS* planar chromatography manager *version 1.4.2.* (CAMAG, Switzerland) having facility of multi wavelength scanning. Drying and concentration steps were performed using rotavapor (Buchi, Switzerland) model no. R-205 equipped with auto vacuum controller (model no. V-800). Ultrsonicator (Enertech, Mumbai, India) used for homogenizing of test and standard solutions.

2.4. Standard sample preparation and calibration curve

A stock solution of quinine was prepared by dissolving 50 mg of quinine in a 25 mL, volumetric flask containing methanol, sonicated for 10 minutes with ultrasonicator. The average value of the peak area was used for calculations after ensuring that the RSD was < 2% for the six replications. A calibration curve was plotted between increasing amounts of quinine per spot and their peak area response. A straight line was obtained between 4.0 to 24.0 µg spot^{-1}. Correlation coefficients (r) were ≥0.9927. The linear regression equation was found to be $y = 1342.15x + 5358.57$, where y is the peak area and x is the concentration.

2.5. Soxhlet extraction and test sample preparation

Soxhlet extraction of 10 g (14 mesh) powdered bark was performed on waterbath with 200 mL of corresponding solvent for 10 hrs. Extract thus obtained was concentrated *in vacuo* via rotavapor and re-dissolved in methanol and volume made up to 100 mL. This solution was taken as test sample for quantification purpose.

2.6. Chromatography

Chromatographic separation of target analyte quinine was performed on 20 cm × 20 cm aluminium-backed HPTLC plates coated with 200 µm layers of silica gel $60F_{254}$ (E. Merck, Darmstadt, Germany). Before use the plates were prewashed with methanol and activated at 110°C for 5 min. Both test and standard samples (5 µL each) were applied on to HPTLC plate as 6 mm wide bands and 12 mm apart from middle of bands by spray-on technique along with nitrogen gas supply for simultaneous drying of bands, by means of a Camag (Switzerland) Linomat V sample applicator fitted with a 100 µL syringe (Hamilton, Bonaduz, Switzerland). A constant spot application rate of 0.15 µL s^{-1} was used. Plates were developed to a distance of 165 mm, in the dark, with 30 mL ethyl acetate - diethyl amine, 88 : 12 (*v* / *v*), as mobile phase. Before development the chamber was

saturated with mobile phase for 15 min at room temperature (25 ± 2°C). Chromatography was performed in camag's twin-trough chamber. Wavelength for detection of quinine was evaluated from complete uv spectrum of quinine (Figure 2). Densitometric scanning was performed with a Camag TLC scanner 3 in reflectance–absorbance mode at 236 nm, under control of Camag *winCATS* planar chromatography manager software (*version 1.4.2*). The slit dimensions were 5 mm × 0.45 mm and the scanning speed was 10 mm s^{-1}. Chromatogram thus obtained showed peak of quinine at R_F 0.24 (Figure 3).

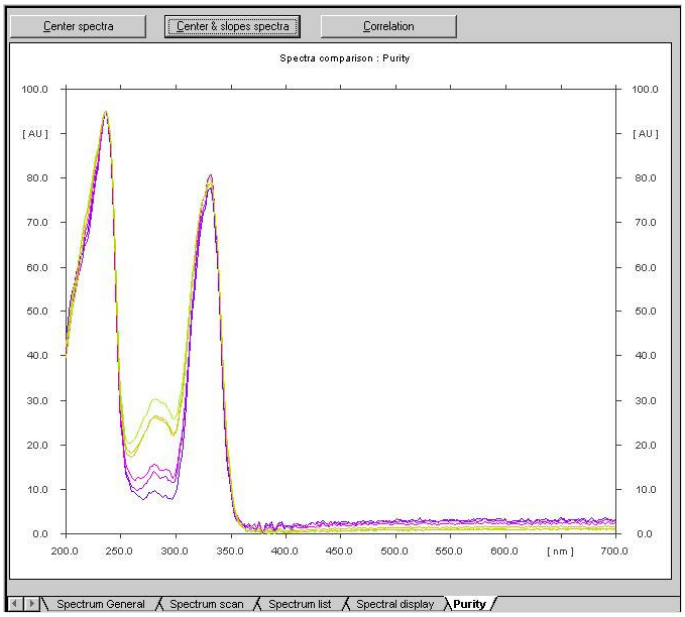

Figure 2. Complete UV spectrum of quinine

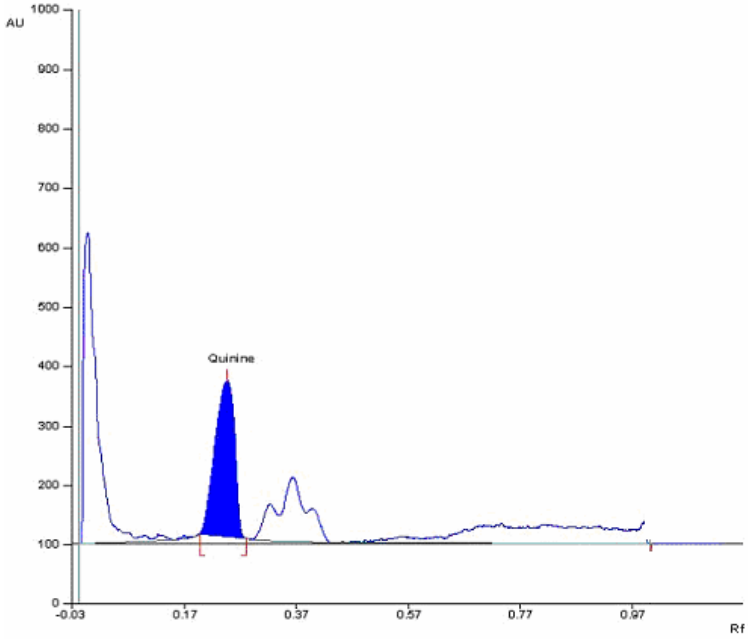

Figure 3. Chromatogram of Quinine separation

3. Results and Discussion

3.1. Screening of solvent for best extraction of quinine

Screening of quinine extraction was performed by using different solvents (*n*-hexane, chloroform, ethyl acetate and methanol) under solid-liquid hot (soxhlet) extraction for 10 hrs. in all cases. Methanol showed good recovery of target analyte quinine (Table 1).

3.2. Effect of basic modifier

As the efficiency of extraction is known to increase in presence of basic media, we have chosen diethyl amine (DEA) for the purpose of modifying the extraction solvent. Different percentages of DEA used to extract quinine in all the four solvents. The results of plain and modified solvent extraction have been summarized in table 1. Methanol modified with 20 per cent (v/v) of DEA was found to be best solvent for extraction although *n*-hexane, chloroform, ethyl acetate and methanol modified with 20 per cent (v/v) DEA yielded 4.88, 3.92, 12.57 and 1.49 times more quinine than that of unmodified solvents respectively. The highest analytical recovery of quinine in the powdered bark was found to be 2.202 % (dry wt. basis), when methanol modified with DEA (20 % DEA, v/v) used for extraction, where as further addition of DEA to methanol (i.e., 30 % DEA, v/v) did not showed remarkable increase in quinine content. *n*-hexane extraction without modification showed 0.372 % of quinine (dry wt. basis), where as unmodified methanol yielded 1.474 %. It concludes that polarity of solvent (solvent with higher dielectric constants) and base are the two main factors influencing extraction of quinine. Thus, solvents modified with 20 % DEA showed best recovery as compared with other unmodified or lesser-modified solvents.

4. Method Validation

4.1. Specificity

The developed HPTLC – UV method was found to be specific as no interfering peak found during detection of quinine as is also evidenced by peak purity data (Table 2).

4.2 Linearity and range

For linearity, five different concentrations of quinine were used in a working range of 4-24 µg per zone. Linear regression equations and correlation coefficient (r) values for test quinine presented in Table 2. The method showed good linearity in the given range.

4.3. Precision (accuracy)

Precision of the method was determined by three replications of each sample. The precision (%RSD) of the replications was found to be less than 2, which is indicative of a precise method. Peaks of quinine eluted on to HPTLC plate are found to be pure (table 2).

4.4. Limit of detection and quantitation (LOD and LOQ)

Limit of detection and quantitation was determined by spotting increasing amounts (16 – 112 ng; n = 2) of standard quinine solution of concentration 16 µg mL^{-1} (4.0 mg of quinine in 250 mL methanol). Based upon signal-to-noise ratio 1 : 3, LOD was found to be 32 ng $spot^{-1}$. LOQ was calculated as 3.34 times of LOD i.e., 106.88 ng $spot^{-1}$ but experimentally LOQ was found to be 96 ng $spot^{-1}$.

Table 1. Optimization of Extraction solvent and conditions

S. No.	Sample I.D.	% Extractive wt.	% Quinine Content	Mean % of quinine content (dry wt. basis) (±SD)	%RSD
1	100% *n*-Hexane	0.95	0.365 0.372 0.380	0.372 % (±0.008)	2.151
2	5% DEA in *n*-Hexane	4.80	1.586 1.612 1.645	1.614 % (±0.020)	1.859
3	10% DEA in *n*-Hexane	5.40	1.822 1.780 1.736	1.779 % (±0.043)	2.417
4	20% DEA in *n*-Hexane	7.20	1.800 1.818 1.824	1.814 % (±0.012)	0.662
5	100% Chloroform	5.40	0.482 0.456 0.457	0.465 % (±0.015)	3.226
6	5% DEA in Chloroform	16.30	1.552 1.538 1.522	1.537 % (±0.015)	0.976
7	10% DEA in Chloroform	17.70	1.597 1.614 1.609	1.607 % (±0.009)	0.560
8	20% DEA in Chloroform	17.80	1.817 1.837 1.820	1.825 % (±0.011)	0.603
9	100% Ethyl acetate	2.70	0.166 0.159 0.162	0.162 % ((0.004)	2.469
10	5% DEA in Ethyl acetate	7.00	1.807 1.810 1.813	1.810 % ((0.003)	0.166
11	10% DEA in Ethyl acetate	8.20	1.981 2.009 1.985	1.992 % ((0.015)	0.753
12	20% DEA in Ethyl acetate	14.70	2.063 2.050 1.994	2.036 % ((0.037)	1.817
13	100% Methanol	16.10	1.434 1.491 1.498	1.474 % ((0.035)	2.374
14	5% DEA in Methanol	21.60	1.813 1.777 1.789	1.793 % ((0.018)	1.004
15	10% DEA in Methanol	22.40	1.892 1.970 1.951	1.938 % ((0.041)	2.116
16	20% DEA in Methanol	24.20	2.193 2.206 2.206	2.202 % ((0.008)	0.363

4.5. Robustness

Robustness of the method was determined by performing small variations in mobile phase ratio (i.e., small variations in DEA volume), height of plate development and TLC tank saturation time. The results indicated insignificant differences in assay and thus indicative of a robust method.

4.6. Calculation of Flow Constant [9]

The flow constant or velocity constant (k) is a measure of the migration rate of the solvent front. It is an important parameter for TLC users and can be used to calculate, for example, development times with different separation distances, provided that the sorbent, solvent system, chamber type and temperature remain constant. The flow constant is given by the following equation:

$$k = \frac{Z_F^2}{t}$$

where, k is flow constant [mm^2/s], Z_F is distance between the solvent front and the solvent level [mm] and t is the development time [s]. The flow constant as calculated by this method was found to be 8.631 $mm^2\ s^{-1}$.

Table 2. Summary of validation parameters of Quinine

Parametrs	**Results**
Linearity	
Range (µg/spot)	4-24
Linear equation	Y = m X + C
Slope (m)	1342.151
Intercept (C)	5358.571
Correlation coefficient (r)	0.99270
Standard deviation (sdv)	5.65 %
Peak purity of eluted test quinine spot	
Correlation coefficient, r (s, m)	0.999103
Correlation coefficient, r (m, e)	0.997273
Peak purity of eluted standard quinine spot	
Correlation coefficient, r (s, m)	0.999045
Correlation coefficient, r (m, e)	0.999012
Precision (%RSD)	
Intra day (n = 3)	
Repeatability of Samples	0.94
Repeatability of peak area	1.97
Repeatability of R_F	1.80
Inter day (n = 3)	
Repeatability of Samples	1.11
Repeatability of peak area	ND*
Repeatability of R_F	1.78
Limit of detection (LOD)	32 ng
Limit of quantification (LOQ)	96 ng
Specificity	specific
Number of theoretical plates, efficiency, (N)	860.77
Flow rate	8.631 $mm^2\ s^{-1}$

*not done

4.7. Calculation of Plate efficiency (N)

Plate efficiency, also known as number of theoretical plates was calculated for the described method by the following equation [10, 11]:

$$N = \frac{16 \times l \times z}{w^2}$$

where, l is the distance (in mm) traveled by solvent front, z is the distance (in mm) traveled by the target spot from application point and w is the width of spot (in mm) in the direction of mobile phase ascending. The plate efficiency was calculated to be 860.77 for quinine.

5. Conclusion

The improved method is useful for 'in process' analytical method for quinine determination as well as for the screening purposes and also provides useful information towards development of extraction technology for processing of quinine. This method represents an improved approach for quinine determination taking into consideration of 'number of theoretical plates' as well as 'flow constant' as parts of validation. It offers the advantages of speed, simplicity and selectivity.

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