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DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY UV-VISIBLE SPECTROMETRY METHOD FOR THE DETECTION OF QUINAPYRAMINE SULFATE IN RABBIT PLASMA

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ABSTRACT

Quinapyramine sulfate (QS) is the most common and effective drug used for treatment of Surra. This is the first report for developing a sensitive high performance liquid chromatographic method (HPLC) UV-visible spectrometry assay for the determination of QS. A simple and robust HPLC method with UV-visible detection for QS in rabbit plasma has been developed. The QS in plasma samples was resolved by reverse-phase chromatography on a Shimadzu-HPLC system equipped with UV/VIS detector using column Inertsil ODS C18, 5 µm (150 x 4.6 mm). Analytes were separated at 1 ml/min with a linear gradient from 40% H_2O/60% acetonitrile/ 0.1% formic acid over 5 min and quantified by UV absorbance at 296 nm. Recoveries at concentration of QS ranged from 2.5 to 100,00,00 ng/ml were observed between 71.45 % and 90.23%. This high performance liquid chromatographic method for the determination of QS in animal plasma can reliably quantitate concentrations of QS as low as 1.7 ng/mL.

INTRODUCTION

Trypanosomosis (Surra), caused by the parasite *Trypanosoma evansi*, is an economically important protozoan disease of livestock. Approximately 35 million doses of trypanocides are administered to domestic ruminants annually at an estimated cost of 35–40 million US dollars (source: Food and Agricultural Organisation of the United Nations (FAO), Rome). The disease is mainly controlled by the trypanocidal drug quinapyramine sulfate (QS).[1] QS

is chemically defined as 4-Amino-6-((2-amino-1, 6-dimethyl-4-pyrimidinyl) amino)-1-methylquinaldinium ion (Fig. 1) The drug acts indirectly by inhibiting protein synthesis through the displacement of magnesium ions and polyamines from ribosomes.[2] With the recent influx of resistance and toxicity of QS, it has become necessary to define a suitable method for controlling and detecting the major constituents of drug in plasma. The activity of drugs has been correlated with their concentrations in whole blood, plasma or serum.[3] In case of UV or visible spectrophotometer method the sensitivity is of order 1-10 µg/ml.[4] The specificity of detection is also often very poor due to the large number of endogenous contaminants in biological fluids.

We previously reported the synthesis and evaluation of quinapyramine sulfate nanoparticles in *T. evansi* animal model.[5] The use of QS in trypanocidal nanoformulations with highly reduced dose of QS necessitates the development of sensitive methods for determining the QS in animal plasma. High performance liquid chromatographic methods have been reported to increase the sensitivity and specificity combined with range of compounds that can be measured, using very low volume of the biological sample (usually< 10µl).[6] The method for estimating the QS levels by HPLC is not well documented. Only a single study was reported in 1985 using silica gel column and a large volume (10ml) of serum sample.[7] The inherent disadvantages like a very high pressure (sometimes up to 250 bars), a lengthy flushing or equilibration time between runs, and limitations on the flow rate, the length of separation column and resistance in the columns necessitate the development of a refined and sensitive method for measuring the QS contents. It was thought pertinent to develop a rapid and sensitive HPLC method for the estimation of QS in plasma. Here, we report for the first time, a sensitive HPLC UV-visible spectrometry assay for the determination of QS. The advantages of the method include small sample volume, single-step procedure, and short run time with low detection limit of QS in the plasma samples.

EXPERIMENTAL

Chemicals and Reagents

QS was procured from Vétoquinol India Animal Health Pvt. Ltd (Mumbai, India). The organic solvents acetonitrile and methanol of HPLC grade were procured from Qualigens Fine Chemicals, Mumbai, India. The analytical reagent grade perchloric acid and potassium dihydrogen orthophosphate were purchased from SD Fine Chemicals, Mumbai, India.

Instrumentation and Chromatographic Conditions.

The Shimadzu HPLC system (LC-2010C HT, Japan) consisting LC-20AT pump and a SIL-20ACHT autosampler, equipped with a SPD-20A UV/VIS detector and SPD-M20A photodiode array detector (PDA) was used. LCsolution version 1.25 software was utilized to process the chromatograms. The analysis was carried out using phenomenex C18 Luna 5µ 100A (250×4.60 mm) column mentioned at 35^{o}C. To detect QS in deionized water (DW), the mobile phase consisted of acetonitrile and 0.3 % perchloric acid in water (20:80 v/v); was pumped at a flow rate of 1.4 ml/min. The injection volume (20 µl) and run time (10 min) were kept constant throughout analysis. The estimation was carried out at a detection wavelength of 297 nm. The detection of QS in plasma samples was resolved by reverse-phase chromatography on a Shimadzu HPLC system equipped with UV/VIS detector and PDA. The analysis was carried out using Inertsil ODS C18, 5 µm (150 x 4.6 mm) column. Analytes were separated at 1 ml/min with a linear gradient from 40% H_2O/60% acetonitrile/ 0.1% formic acid over 5 min. and quantified by UV absorbance at 296 nm.

Preparation of Calibration Standard

The stock solution of QS (1 mg/ml) prepared in distilled water was further diluted to give calibration standards of QS at concentrations ranging from 0.50, 1, 2.5, 5, 10, 15, 25 and 50 ng/ml by spiking appropriate amount of the standard solution in plasma.

Calibration Standard

For HPLC analysis, 1 mg of the QS was accurately weighed using an analytical balance (readability 0.01 mg). The weighed sample was dissolved in 1 ml mobile phase. Aliquots of the stock solution were serially two fold diluted with mobile phase. The different dilutions were used to produce calibration solutions of concentrations ranging from 1.75 to 100000 ng/ml.

Sample Preparation for Analysis

Plasma was separated from rabbit blood collected in EDTA tubes, by centrifuging at 3000 rpm for 10 min and was stored at -20°C. The samples were diluted with mobile phase just before use. The sample was prepared by adding 1ml of plasma containing QS (calibration standard) into the mobile phase. The contents were vortexed, centrifuged at 4000 rpm for 15 min at 4°C and finally the supernatant was collected for further use in HPLC column.

Validation of Method

Linearity

The linearity of the assay was determined by plotting a standard curve over a concentration of QS ranges from 2.5 ng to100,00,00 ng/ml. A series of dilutions were prepared from the stock solution of QS 1 mg/ml in the mobile phase: acetonitrile-water-formic acid (60:40:0.1 %v/v). The data from peak area versus drug concentration plots were used for linear least square regression analysis.

Precision and Accuracy

The precision (as the measure of repeatability) of the analytical procedure assessing the chemical purity of QS was tested by preparing 6 individual model solutions with impurities at the specification limit. Every model solution was analyzed in triplicate; the relative standard deviation (RSD) for all 18 analytes was calculated for each impurity peak area. To investigate the unknown impurities, sample solutions of QS were prepared by diluting the stock solution to their specification limit. The inter day variation in the peak area of the drug solution was calculated in terms of coefficient of variation (CV) and obtained by multiplying the ratio of standard deviation to the mean with 100 [CV=±SD/mean X 100] as shown in. **Table 1.**

To verify the closeness of agreement between the expected value and the value observed, the accuracy of the method was evaluated by determination of recovery of QS at different levels of concentrations and expressed in terms of percent recovery.

RESULTS AND DISCUSSION

QS an aminoquinaldine, is effective in treatment of an economically important disease trypanosomosis (Surra) caused by the parasite *T. evansi.* It is a major constraint to livestock productivity in Asia, Africa and South America. Despite of an economically important disease, it is considered as neglected disease. No work or method has been reported from the last thirty years for estimating the QS levels. This is the first report for developing a sensitive HPLC UV-visible spectrometry assay for the determination of QS. It describes a highly sensitive, precise, and reproducible HPLC method for the determination of QS in plasma. The procedure for sample preparation is rapid and inexpensive.

The effect of various parameters was undertaken by varying one parameter at a time and controlling all other parameters. We carried out number of experiments to get base line separation of the components and sharp peaks, by varying different components like

percentage of organic phase in the mobile phase, pH of the aqueous phase, pH of the selected mobile phase and flow rate by changing one at a time and keeping all other parameters constant. Initially, we optimized the parameters using quinapyramine sulfate dissolved in DW, the retention time of QS was 6.4 min. While detecting QS in rabbit plasma, acetonitrile:water:formic acid (60:40:0.1 % v/v) was chosen as the optimum mobile phase. The retention time of 2.05 ± 0.1 min was observed at 296 nm UV absorption maxima. Representative chromatograms of QS in water and plasma are shown in Fig. 2A & B. No interference from endogenous components or QS metabolites was observed in plasma. The baseline was relatively free from drift.

To test whether QS has been linearly eluted from the column, different amounts of QS were taken and analyzed by the above mentioned procedures. The linearity of the assay determined by plotting a standard curve over a concentration range of 2.5 ng –100,00,00 ng/ml of QS showed correlation coefficient (R^2) of more than 0.9770 and considered as acceptance criterion. The results are summarized in Table 1. The peak area ratios of component areas are graphically represented illustrating the linear fit of the system in **Fig.3.** The polynomial regression for the calibration plots showed good linear relationship with coefficient of correlation (0.99)' $Y= 19798x^2+ 99216x+ 90841$ (n = 8) over the concentration range studied. The range of reliable quantification was set at 0.01–1000 µg/ml as no significant difference was observed in the slopes of the standard curves in this range. The linear regression data for the calibration plot is indicative of a good linear relationship between peak area and concentration over a wide range. The low values of the standard deviation, the standard error of slope, and the intercept of the ordinate showed that the calibration plot did not deviate from linearity.

The accuracy of the method was evaluated by determination of recovery of QS at different levels of concentrations and expressed in terms of percent recovery as shown in Table 2. Recoveries at concentration starting from 2.5 to 100,00,00 ng/ml ranged from 71.45 % to 90.23% respectively. The very low quantification limit obtained with a UV detector allowed us to avoid using HPLC-with fluorometric detection which requires more expensive equipment, and makes this method useful for pharmacokinetic studies. UV detectors provide more reproducible and stable results than fluorometric detectors.[8] Moreover, the use of an isocratic mobile phase consisting of simple composition gives the column a longer lifetime and reduces the risk of protein precipitation associated with the use of solvent system.[9] The

developed method can be used extensively for measuring QS in the plasma in pharmacokinetic study.

Table 1: Linearity of quinapyramine by HPLC-UV

Linearity Level (%)	Concentration (ng/ml)	Peak Area	CV (%)
10	2.5	1026743	2.040342
50	5	1205660	0.834422
100	10	1345955	0.650991
150	25	1560848	1.11364
200	100	1836049	0.839797
250	1000	2166203	0.697438
300	100,00	2555320	1.204756
350	100,00,00	2797568	2.440806

Table 2:Percent recovery of quinapyramine by HPLC-UV

Sr. No	Measured Concentration (ng/ml)	Expected drug Concentration (ng/ml)	% Recovery (n=3)
1	1.78	2.5	71.45
2	3.66	5	73.21
3	7.76	10	77.64
4	19.84	25	79.39
5	81.34	100	81.34
6	865	1000	86.57
7	8734	100,00	87.34
8	902300	100,00,00	90.23

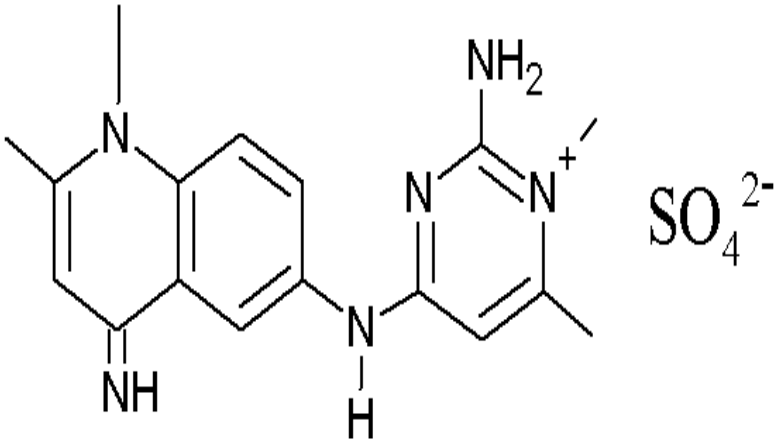

Fig 1. Chemical structure of Quinapyramine sulfate

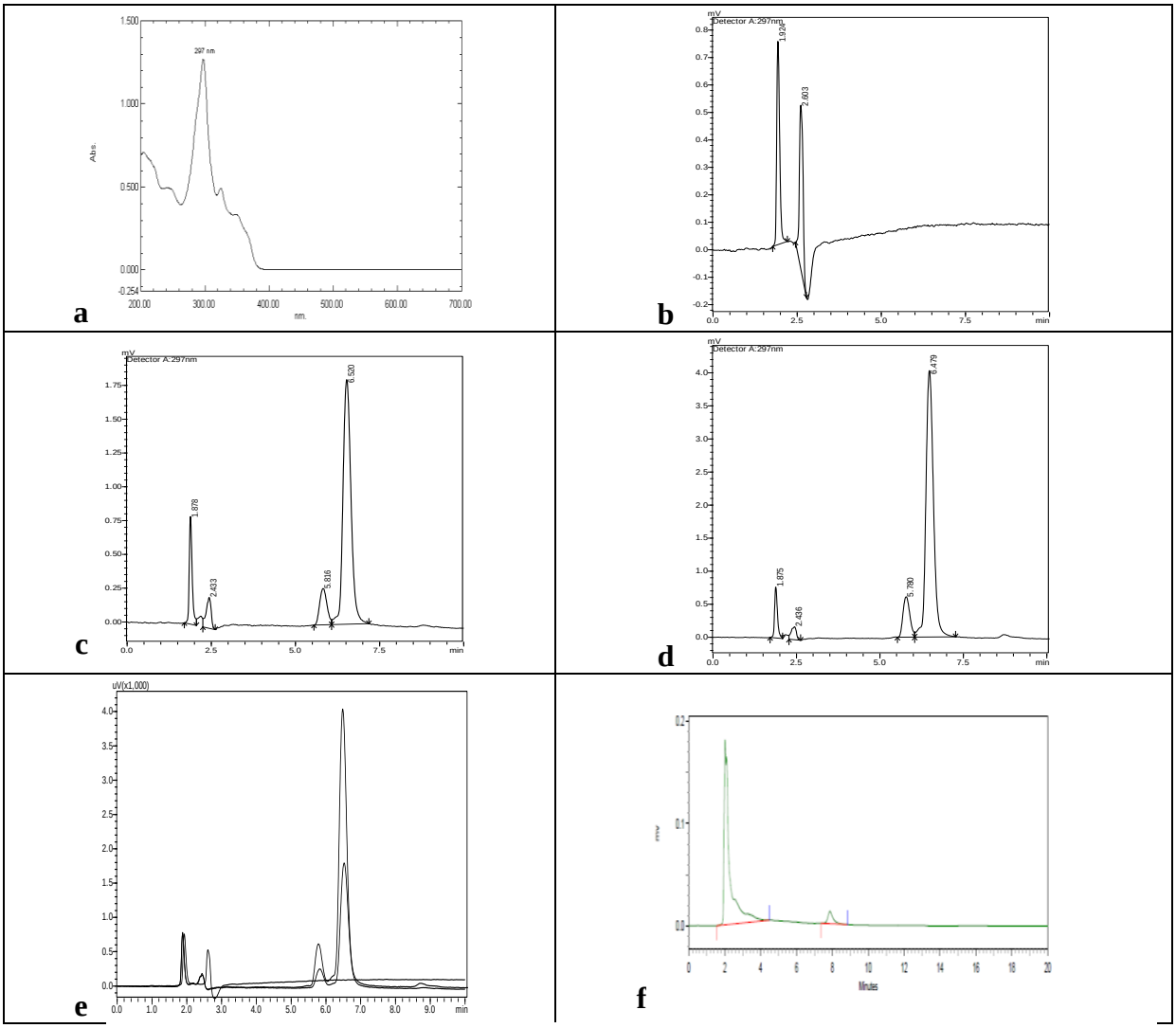

Fig. 2: (a) UV spectrum of Quinapyramine sulfate (QS) in DW, HPLC chromatogram of (b) blank, (c) QS 0.5 µg/ml, (d) QS 1 µg/ml and (e) overlay of b,c and d (f) QS in rabbit plasma. X-axis: Time in min Y-axis: Absorbance

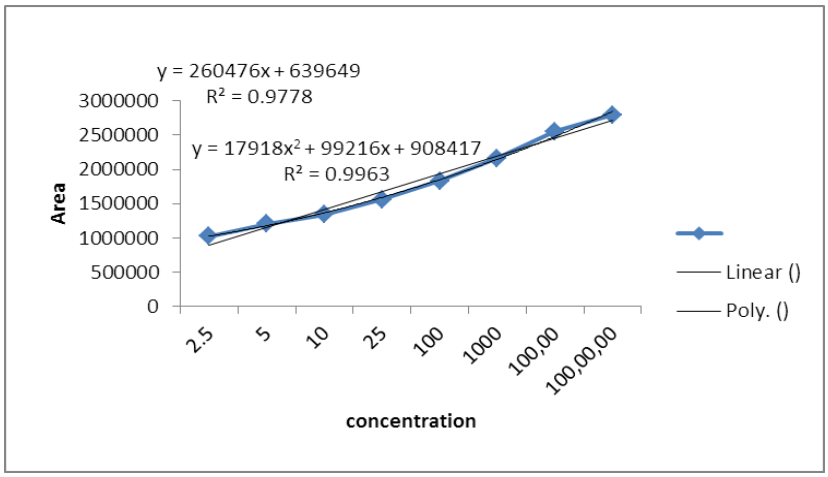

Fig 3. Graphical representation of area versus concentration of drug (ng/ml) in rabbit plasma

CONCLUSION

We report for the first time a simple, rapid, reproducible, and sensitive HPLC-UV method for analysis of QS in plasma samples. It describes the use of small sample volume, easier single step procedure, and shorter reaction time with low detection limit of QS in plasma samples. The method was validated for analysis of QS in plasma samples over the range 2.5 – 1000, 00 ng/ml.

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