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Development and validation of the simultaneous determination of artemisone, clofazimine and decoquinate with HPLC

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The aim of this study was to develop and validate a novel HPLC method for the simultaneous analysis of artemisone, clofazimine and decoquinate. Detection was obtained at two wavelengths; 284 nm (clofazimine) and 210 nm (artemisone and decoquinate). Gradient elution was used with mobile phase A (**A**) consisting of 0.005 M sodium octanesulphonic-acid (pH 3.5) and mobile phase B (**B**) of HPLC grade acetonitrile. The flow rate was set to 1.0 ml/min with (**A**) at 35% and (**B**) at 65% for 2 min, followed by a gradient shift of 10/90% ((**A**)/(**B**)) over a duration of 4 min. After 10 min, the initial gradient conditions were readjusted to 35/65% ((**A**)/(**B**)). Distinctive peaks were identified for clofazimine, artemisone and decoquinate, respectively. The proposed HPLC assay method was validated and found to be reliable, reproducible and accurate for simultaneous analysis of the three compounds.

1. Introduction

Tuberculosis (TB) poses a significant public health threat, with 20 – 40% of the world's population being affected. Less than 14% of TB cases are extra-pulmonary, of which only 1.0 – 1.5% manifests as cutaneous tuberculosis (CTB). CTB is quite an exceptional presentation of TB; resulting in it being undefined and often misdiagnosed (Bravo and Gotuzzo 2007; Rullán et al. 2012; Carman and Patel 2014; Galagan et al. 2014).

A fixed-dose combination of artemisone, clofazimine and decoquinate formulated in a topical dosage form was chosen as a possible therapy to effectively treat CTB. The combination of these three active pharmaceutical ingredients (APIs) was based on the combination strategy of oxidant and redox APIs for the treatment of malaria and TB. The combination of the three APIs formed part of an investigative study; since the effectivity of decoquinate against TB has not yet been established, but its lipophilicity renders it an attractive compound for assessment. Although clofazimine was considered ineffective against pulmonary TB, recent advances in technology have renewed its use for TB treatment, and therefore it was included in this combination as an API with redox capabilities. Artemisone is effective against *Plasmodium falciparum* (malaria), but was included in this study as an oxidant API (Cholo et al. 2011; Steyn et al. 2011; Haynes 2013).

Clofazimine ($C_{27}H_{22}Cl_2N_4$) (Fig. 1A) (modified from Cholo et al. 2011) has an aqueous solubility of 10 mg/L, a log P of 7.60, a pKa of 8.51, a melting point of 210 – 212 °C and a molecular weight of 473.40 g/mol (Holdiness 1989; Brittain and Florey 1992; Cholo et al. 2011; Bolla and Nangia 2012; Srikanth et al. 2014). Artemisone ($C_{19}H_{31}NO_6S$) (Fig. 1B) (modified from Biamonte et al. 2013), in addition, has an aqueous solubility of 89 mg/L (pH 7.2, water), a log P of 2.49, a melting point of ≈199 °C and a molecular weight of 401.52 g/mol (Nagelschmitz et al. 2008; Dunay et al. 2011; Steyn et al. 2011). Very little information is available for decoquinate, since it has mainly been used as a veterinary API. Decoquinate ($C_{24}H_{36}NO_5$) (Fig. 1C) (modified from Biamonte et al. 2013) has a log P of 7.80, a pKa of 10.76, a melting point of ≈219 °C and a molecular weight of 417.54 g/mol (Nam et al. 2011; Iglesias et al. 2014). Currently, no HPLC method for the simultaneous determination of these three compounds is available in literature. Therefore, this method was developed and validated to determine the concentration assays of the three compounds simultaneously for different routes of administration such as solid oral dosage forms or transdermal formulations. This method was developed and validated based on the International Conference on Harmonisation (ICH) and current Good Manufacturing Practice (cGMP) guidelines and parameters (ICH 2005; FDA, 2011).

Table 1: Solubility (µg/ml) (37 °C) determined for artemisone, clofazimine and decoquinate in nine different solvents

	Artemisone	Clofazimine	Decoquinate
Water	100.50	0.05	0.00
PBS (pH 7.4)	86.01	0.00	1.21
Trisaminomethane (pH 7.4)	99.07	0.00	0.00
Ethanol	44 600.00	1 824.70	181.70
Methanol	46 541.60	827.74	163.13
Isopropanol	26 351.00	1 018.53	160.08
Acetone	105 923.00	2 181.64	18.95
Acetonitrile	201 960.00	1 207.80	12.98
THF	194 190.00	143 410.00	241.13

2. Investigations, results and discussion

The proposed method was validated in terms of linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), system suitability and robustness. Thereafter the solubility of all three APIs was determined in different solvents (Table 1). The solubility of decoquinate, when compared to the other two APIs, was the lowest in all investigated solvents, consequently its solubility was used as the deciding parameter during the method development steps. The solubility of decoquinate in tetrahydrofuran (THF) was the highest, however, since it is considered toxic, it will not be the solvent of choice during pre-formulation, dosage form development or API release testing from either solid or semi-solid formulations. Hence, ethanol was chosen as the main solvent.

The linearity was determined by constructing a regression plot of API concentration versus peak area response, allowing the calculation of

Table 2: Obtained validation parameters for the three compounds

Linearity	Artemisone (n = 9)	Clofazimine (n = 7)	Decoquinate (n = 9)
Concentration range (µg/ml)	5.00 – 400.10	0.93 – 55.86	1.50 – 120.10
Regression equation	y = 1.079x - 0.375	y = 72.623x + 42.390	y = 23.261x + 51.866
Correlation coefficient (r^2)	0.9999	0.9937	0.9989
Accuracy			
Mean recovery (%) (%RSD)	99.9 (± 1.4)	100.0 (± 0.3)	99.3 (± 7.5)
Stability			
Max sample deviation from hour zero (%) (%RSD)	2.5 (± 0.8)	0.2 (± 0.5)	5.0 (± 1.1)
System suitability			
Retention time (min) (%RSD)	7.5 (± 0.1)	6.4 (± 0.3)	10.2 (± 0.1)
Peak area (%RSD)	23.19 (± 0.16)	430.76 (± 0.90)	187.10 (± 1.71)

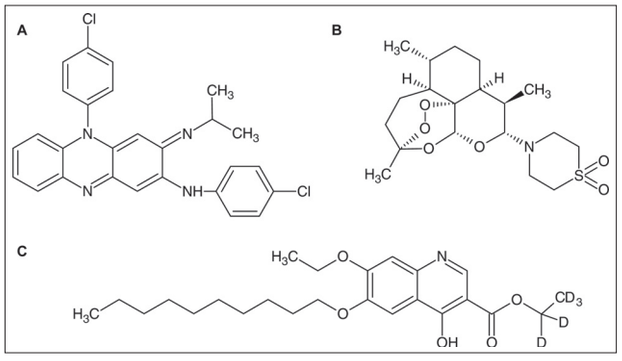

Fig. 1: Molecular structures of A) clofazimine, B) artemisone and C) decoquinate.

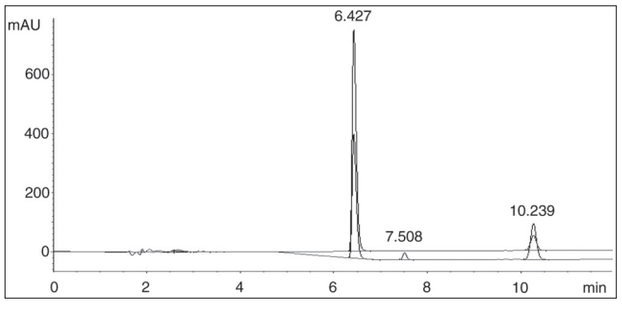

Fig. 2: Chromatographs of a standard solution containing clofazimine, artemisone and decoquinate, respectively. The top chromatogram signifying detection obtained at 284 nm and the bottom chromatogram showing detection at 210 nm.

a regression equation for each API. The resulting equations are listed in Table 2. The correlation coefficient (r^2) was also determined, where the strongest linear relationship is indicated by a correlation coefficient of 1 (Krause 2003; UNODC 2009). The accuracy of the three compounds can be seen in Table 2. The mean recovery percentages were all between 98 and 102 % (% RSD < 15 %), thus complying with validation requirements for accuracy parameters.

Precision was conducted during a three-day period at three concentration levels (Table 2). All the parameters mentioned adhered to the specifications and thus the method was found to be accurate and precise. The stability of artemisone, clofazimine and decoquinate was evaluated, and no significant instability was observed for at least 24 h (Table 2). None of the API concentrations deviated with more than 15% and were consequently found to be stable for at least 24 h after preparation. System suitability was determined with the injection precision for retention times and peak areas as depicted in

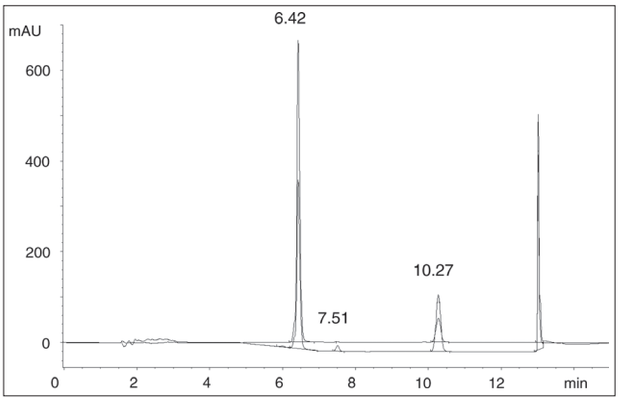

Fig. 3: Chromatographs obtained with a solution containing typical excipients used in formulation of solid oral dosage forms, observing clofazimine, artemisone and decoquinate, respectively. The top chromatogram signifying detection obtained at 284 nm and the bottom chromatogram showing detection at 210 nm.

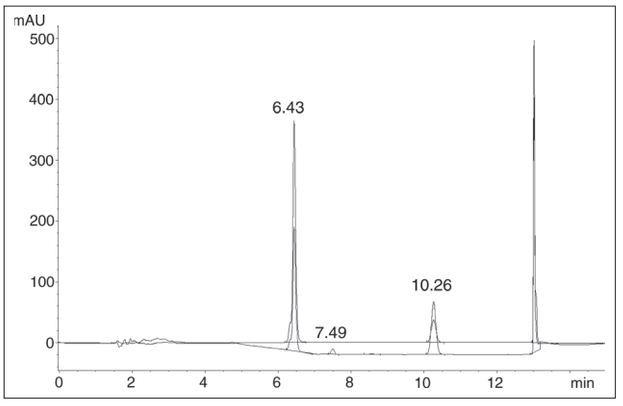

Fig. 4: Chromatographs of excipient solution for transdermal/topical delivery systems showing clofazimine, artemisone, and decoquinate, respectively. The top chromatogram signifying detection obtained at 284 nm and the bottom chromatogram showing detection at 210 nm.

Table 2. System suitability was determined from six replicate injections. The obtained peaks were analysed in terms of peak area and retention times. All %RSD values were less than 2% (Table 2) and as a result the method was found to be suitable for the HPLC system. The LOQ is the lowest concentration of API that can be quantitatively ascertained, above which analysis is possible with the specified degree of accuracy and precision. LOQ is used particularly for determining

Table 3: Precision data for artemisone, clofazimine and decoquinate

		Artemisone	Clofazimine	Decoquinate
Concentration (µl/ml)		393.0	74.0	128.0
Repeatability (intra-day)	%RSD	1.03	0.21	4.55
	Mean recovery (%)	99.86	99.99	99.57
Intermediate precision (inter-day)	%RSD (day 2)	0.78	0.17	3.68
	Mean recovery percentage (day 2)	99.96	100.01	99.67
	%RSD (day 3)	1.20	0.14	4.33
	Mean recovery percentage (day 3)	99.89	99.99	99.63
	p-value of ANOVA (for the three days)	0.938	0.958	0.862

impurities and/or degradation products (ICH 2005; VICH 2015). To ensure whether LOQ is accurate, the API should be injected at the calculated LOQ concentration (n = 6) and the %RSD should be calculated for each concentration. RSD variation should not exceed 20 % (Krause 2003; ICH 2005; Westgard 2008; Huber 2010; VICH 2015; González et al 2014). The LOD, on the other hand, can be determined according to several methods, though the method used in this study is based on the standard deviation of the regression line or the y-intercept and the slope of the calibration curve. The calculated LOD was 1.98 µg/ml for artemisone, 3.11 µg/ml for clofazimine and 2.29 µg/ml for decoquinate. Confirmation of the LOD required injecting the samples at the calculated concentration six times. The experimental LOD obtained was thus 4.42 µg/ml (%RSD 19.68), 0.042 µg/ml (%RSD 17.73) and 0.703 µg/ml (%RSD 17.79) for artemisone, clofazimine and decoquinate, respectively. LOQ was subsequently determined as 13.39 µg/ml for artemisone, 0.13 µg/ml for clofazimine and 2.13 µg/ml for decoquinate. The experimentally determined LOD and LOQ values corresponded well; whereas the calculated LOD did not correlate with the experimental values. In our experience the calculated LOD rarely correlates with experimentally determined values and is of limited use.

Since there is currently no dosage form containing this combination of compounds available on the market, it was decided to make two solutions containing possible excipients that is normally included in a solid oral dosage form and a transdermal/topical delivery system, in order to test the sensitivity and selectivity of the method. The solution for solid oral dosage form contained the three APIs, talc, microcrystalline cellulose, polyvinylpyrrolidone (PVP 30), lactose, magnesium stearate, vinylpyrrolidone-vinyl acetate copolymer (Kollidon® VA64) and Pluronic® F-127 dissolved in absolute ethanol in unspecified concentrations. The solution prepared for transdermal/topical delivery contained the three APIs, polysorbate 20 (Tween® 20), polysorbate 80 (Tween® 80), sorbitan monostearate (Span® 60), phosphatidylcholine, cholesterol, safflower oil and olive oil dissolved in absolute ethanol in unspecified concentrations. As observed from Figs 2 – 4 the three compounds delivered peaks at the determined retention times with little to no interference from the different excipients, which can possibly be used during formulation of solid oral dosage forms and transdermal/topical delivery systems. In addition to the novelty for the simultaneous quantification of artemisone, clofazimine and decoquinate the method is reliable and sensitive which complies with the ICH guidelines for method validation. Validation parameters such as linearity, limit of detection and quantitation, accuracy, precision, sample stability and system suitability were established. The reproducible method was also used to determine sensitivity of the method when applying it to an excipient mixture for a possible solid oral dosage form and for a transdermal/topical delivery system. The method was found to be adequate in quantifying the three APIs with virtually no interference.

3. Experimental

An Agilent® 1100 Series HPLC which was used during this study consisted of an Agilent® 1100 pump, diode array detector, and an autosampler injector module, and ChemStation Rev. A.10.02 software was utilised for data acquisition and analysis (Agilent Technologies, Palo Alto, CA). A Restek Ultra C_{18} fully endcapped reversed phase column (250 x 4.6 mm, 5 µm) was used with 100 Å pores, a 20% carbon load (Restek corporation, Bellefonte, US), a pH range of 2.5 – 8.0 and a temperature limit of 80 °C. Gradient elution was used with mobile phase A (**A**) consisting of 0.005 M sodium octanesulphonic-acid (pH 3.5) and mobile phase B (**B**) comprising of HPLC grade acetonitrile. The flow rate was set to 1.0 ml/min with (**A**) at 35% and (**B**) at 65% for 2 min, followed by a gradient shift of 10/90% ((**A**)/(**B**)) over a duration of 4 min. After 10 min the initial gradient conditions were readjusted to 35/65% ((**A**)/(**B**)). An injection volume of 20 µl was used and the UV detection was set at 210 and 284 nm. Retention times were approximately 6.4 min, 7.5 min and 10.2 min for clofazimine, artemisone and decoquinate, respectively and the run time was set to 15 min. Ethanol absolute (99.7%) was used as the solvent throughout the method validation.

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