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A new approach to evaluate stability of amodiaquine and its metabolite in blood and plasma

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Abstract

A stability study for amodiaquine (AQ) and desethylamodiaquine (AQm) in whole blood and plasma is reported. AQ, AQm and chloroquine (CQ) were simultaneously analysed and the ratios AQ/CQ and AQm/CQ were used to ensure correct interpretation of the stability results. CQ was stable in whole blood and plasma at all tested temperatures enabling it to be a stability marker in stability studies. Simultaneous analysis of compounds, of which at least one is already known to be stable, permits a within sample ratio to be used as a stability indicator. The new approach significantly reduced bias when compared to the traditional approach. AQ and AQm were stable in plasma at −86 °C and −20 °C for 35 days, at 4 °C for 14 days and at 22 °C for 1 day. AQ and AQm were stable in blood at −86 °C and 4 °C for 35 days, at −20 °C and 22 °C for 7 days and at 37 °C for 1 day.

Keywords: Antimalarial; Amodiaquine; Chloroquine; Desethylamodiaquine; Liquid chromatography; Solid-phase extraction; Stability

1. Introduction

Amodiaquine (AQ) is a 4-aminoquinoline antimalarial drug that recently has gained renewed interest. The use of AQ for treatment has during the last 15 years been limited after reports of fatal adverse drug reactions in the mid-1980s [1–3]. Lately, several clinical trials have demonstrated the safety and efficacy of AQ when used for treatment [4,5]. As chloroquine (CQ) resistance is becoming more widespread the need for cheap second- and third-line treatments increases.

The stability of an analyte under various conditions is an important parameter in bioanalytical method validation. It is important to evaluate the stability for all conditions encountered from sampling to analysis [6]. The extent of stability studies included in the validation depends on the field of application for the method. The long-term stability of an analyte in the biological matrix should be evaluated for the storage conditions to be used for study samples. The stability must be proven over at least the time from the first sample collection to the last day of analysis. When a stability study is to be conducted it is important that it is carefully planned. All sources of errors should be assessed and minimised in order to get reliable results. FDA guidelines require that at least three aliquots at a low and a high concentration be analysed at three separate occasions. FDA states:

> "All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations. The storage time in a long-term stability evaluation should exceed the time between the date of the first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the

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appropriate concentrations from the first day of long-term stability testing." [7].

This approach suffers from a number of errors originating both from the established between-run variation (affecting the calibration curve to vary) and the various preparation errors (i.e. weighing of reference substance, preparation of stock solution, preparation of working solutions and preparation of calibration standards). An alternative strategy is proposed that can be used in addition to the above for correct interpretation of stability. A previous reported assay permits simultaneous determination of AQ, CQ and their main metabolites in capillary blood applied onto sampling paper [8]. This assay was modified to permit analysis of whole blood and plasma samples and was then validated. CQ has previously been reported stable in plasma and blood cells for at least 3 months [9]. If the stability study samples are spiked with AQ, its metabolite (AQm) and CQ, the ratio between AQ and CQ or AQm and CQ can be used as a marker of the stability. This approach will eliminate all between day assay variation and variation associated with the preparation of new stock solutions and calibration standards. The only source of error not eliminated would be the actual within-run variation (i.e. the within-run variation in the ratio of AQ or AQm to CQ) as all samples originally were spiked at a constant ratio.

2. Materials and methods

2.1. Chemicals

AQ and desethylamodiaquine (AQm) were obtained from Park-Davis Division of Warner-Lambert (Ann Arbor, MI, USA) and CQ and the internal standard (I.S.) were obtained from Sterling-Winthrop (Hertfordshire, UK). The structures are shown in Fig. 1.

Acetonitrile and methanol were of HPLC grade (CHROMASOLV®) from Sigma (St. Louis, MO, USA). Deionised water obtained from a Milli-Q deionised water system (Millipore, Bedford, MA, USA) was used to prepare buffer solutions. The phosphate buffers (0.1 M) were prepared by mixing different amounts of sodium hydroxide and *ortho*-phosphoric acid with Milli-Q deionised water. A concentrated stock solution of sodium perchlorate (1.0 M) was prepared in deionised water.

2.2. Instrumentation

The chromatographic (HPLC) system consisted of a Waters 515 HPLC pump (Waters, Milford, MA, USA), an SP 8880 auto sampler (Spectra Physics, San José, CA, USA) and a Jasco UV-970 Intelligent UV/VIS detector (Jasco, Tokyo, Japan) set at 342 nm. Data acquisition was performed using Chromatography station for WINDOWS 1.7 (DataApex Ltd., Prague, The Czech Republic). The mobile phase was acetonitrile:phosphate buffer (0.1 M, pH 2):sodium perchlorate (1.0 M) (13:86:1, v/v), with a flow rate of 1.0 mL/min through a Zorbax SB-CN column, 5 μm (250 mm × 4.6 mm i.d.), (Zorbax, Inc., Wilmington, NC, USA), protected by a short guard column SecurityGuard C18, 5 μm (4 mm × 3 mm), (Phenomenex, Torrance, CA, USA).

Solid-phase extraction (SPE) was carried out on an automated SPE system, ASPEC XL (Gilson, Middleton, WI, USA) using CBA weak cation-exchange extraction columns containing carboxylic acid as support (1 mL, 100 mg, IST Ltd., Hengoed, Glamorgan, UK). The ASPEC system uses a positive air pressure instead of vacuum to get the liquids through the columns.

2.3. Preparation of stability samples and calibration standards

Concentrated stock solutions of AQ, AQm, CQ and I.S. (approx. 500 μM) were prepared in deionised water. Working solutions containing AQ, AQm and CQ (50 and 5 μM) were prepared in deionised water. Stability samples at a low (300 nM) and a high (3000 nM) concentration were prepared at the start of the stability study by adding appropriate amounts of the

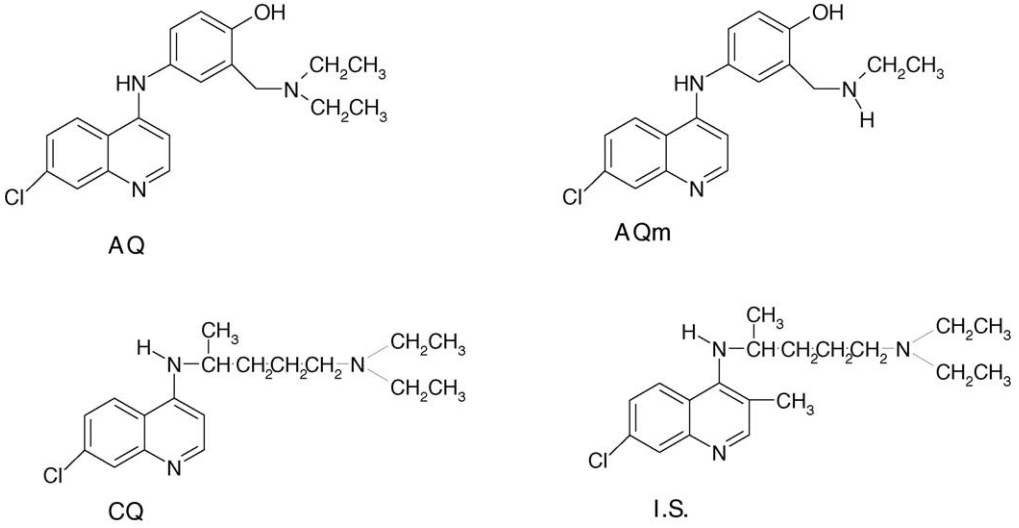

Fig. 1. Structures of AQ, AQm, CQ and I.S.

working solutions (containing AQ, AQm and CQ) to whole blood or plasma. The stability samples were stored in 250 μL aliquots at assigned temperatures until analysed.

New stock solutions of AQ, AQm and CQ (approx. 500 μM) were prepared at every predetermined time point for analysis by dissolving 6–8 mg of each drug in deionised water using a 25 mL volumetric flask. Working solutions containing AQ, AQm and CQ (50 and 5 μM) were thereafter prepared in deionised water and appropriate amounts added to venous blood or plasma (sodium heparin as anticoagulant) to yield six different calibration standards ranging from 100 nM to 4000 nM. All samples were equilibrated at room temperature for 30–40 min before divided in aliquots.

2.4. Analytical procedure

All samples were treated according to a modified version of an earlier published procedure for the simultaneous determination of AQ, AQm, CQ and CQm in dried blood spots [8]. The modified method had within-run and between-run precisions less than 15% in accordance with FDA guidelines [7]. Briefly, 250 μL deionised water was added to 250 μL blood or plasma and the samples were mixed for 10 s. The samples were left undisturbed for 5 min where after 500 μL I.S. (1000 nM) in phosphate buffer (0.1 M, pH 7) was added. The samples were centrifuged at about 20,000 × *g* and the liquid phase was loaded onto a CBA SPE column. The SPE columns were activated and conditioned with methanol and phosphate buffer pH 7. The columns were washed with acetonitrile–phosphate buffer 0.05 M, pH 7 (40:60, v/v) and the analytes eluted from the columns with methanol–formic acid (98:2, v/v). The eluates were evaporated to dryness at 70 °C under a stream of air, reconstituted in 250 μL acetonitrile–hydrochloric acid 0.01 M (10:90, v/v), whereby 100 μL was injected into the HPLC-system.

2.5. Stability

This paper evaluates the stability for AQ and AQm in spiked whole blood and spiked plasma at various temperatures. Whole blood and plasma samples at a low and a high concentration were stored at −86 °C, −20 °C, 4 °C, 22 °C and 37 °C. The whole blood and plasma was spiked with AQ, AQm and CQ at 300 nM and 3000 nM at the start of the stability study. Triplicates from each temperature storage group were analysed together with six freshly prepared calibration standards containing AQ, AQm and CQ (prepared from fresh stock solutions) at four occasions during 0, 1, 3 and 7 days (22 °C and 37 °C) and 0, 1, 2 and 5 weeks (−86 °C, −20 °C, 4 °C). The stability was evaluated by two different approaches. For the traditional approach, peak-height ratios of AQ, AQm and CQ to I.S. (I.S. added during sample preparation) against concentration with non-weighted linear regression (using freshly prepared calibration curves) were used for quantification. Concentrations calculated on each occasion were compared to the back-calculated concentrations at day 0 to assess the stability. The analyte was considered unstable if the concentration had dropped with more than 15%. This cut-off was chosen in accordance with FDA guidelines that accept ±15% difference for quality control samples during routine analysis [7]. The new approach used the fact that all stability samples originally contained AQ, AQm and CQ at a fixed ratio. The ratio between AQ or AQm to CQ within each sample obtained on each occasion were compared to initial ratios at day 0 to assess the stability. The analyte was considered unstable if the ratio had dropped with more than 15%.

3. Results and discussion

The design of long-term stability studies have been extensively discussed by Dadgar et al. [6,10]. Traditionally, QC samples are prepared and stored under the same conditions as potential study samples. Replicates of the QC samples are analysed together with a freshly prepared calibration curve at day zero and after specified periods of time. Concentrations determined on each occasion are compared to establish stability. This approach suffers from a number of errors originating both from the established between-run variation and the various preparation errors. Two different strategies are proposed by Dadgar and Burnett to minimize these errors [6]. The first approach is to prepare batches of QC samples at two concentrations and store them under the required conditions. After the specified period of time, new batches of QC samples at the same concentrations are prepared. Ten replicates of each of the four batches are analysed simultaneously at one occasion. The responses are determined and a mean value with confidence interval can be calculated for each batch. This approach eliminates the between-run variation and also bias associated with the use of different calibration curves. However, it does not eliminate errors due to the two separate preparations. The second approach also starts with the preparation of a batch of QC samples at an appropriate concentration. This batch is subdivided and one half is stored under the required condition while the other half is stored at −130 °C or ideally in liquid nitrogen (nominally at ≤−196 °C). This half can be considered as freshly prepared samples since studies have shown that even highly unstable drugs (e.g. acetylsalicylic acid) are stable for several months in biological matrices at these temperatures [10].

3.1. Stability

Both AQ and AQm have previously shown to be stable in blood dried onto sampling paper at various temperatures [8]. Mihaly et al. have reported that AQ is very unstable to light and adsorbs readily to glass [11]. Stability was not evaluated but AQ seemed to be stable in plasma calibration samples stored at −20 °C for at least 8 weeks. Winstanley et al. modified the method by Mihaly for the analysis of AQ and AQm in plasma, urine and whole blood [12]. All samples were stored at −20 °C protected from light until assayed and no stability problems were mentioned. A method reported by Pussard et al. used the same storage conditions, i.e. −20 °C for whole blood, and reported nothing about stability problems [13]. A storage-stability study performed by Mount et al. showed that AQm and 2-hydroxydesethylamodiaquine were stable in blood at 5 °C for at least 16 weeks [14]. Lately, some contradictory results were published

by Minzi et al. [15]. It reported that AQ and AQm were unstable in spiked whole blood at all tested temperatures (i.e. between −20 °C and 37 °C). The degradation patterns however makes one question whether the results are reliable. AQm for instance was reported to be stable in plasma and urine at −20 °C for 250 days but suddenly degraded by about 50% after another 250 days.

The peak-height ratios of AQ and AQm to I.S. against concentration with non-weighted linear regression were used for quantification according to the traditional approach. Concentrations calculated on each occasion were compared to the back-calculated concentrations at day 0 to assess the stability. The new approach is using the ratio between AQ or AQm to CQ within each sample instead of the concentration. Ratios obtained on each occasion were compared to initial ratios at day 0 to assess the stability. The stability of AQ and AQm (mean value of triplicates) in blood (3000 nM) versus time for the different storage conditions using the traditional approach is shown in Fig. 2. Both AQ and AQm were stable in blood at −86 °C, 4 °C and 22 °C but not at −20 °C or 37 °C over the tested period. The stability of AQ and AQm (mean value of triplicates) in plasma (3000 nM) versus time for the different storage conditions using the traditional approach is shown in Fig. 3. AQ was stable in plasma at −86 °C, −20 °C, 4 °C and 22 °C but not at 37 °C over the tested period. AQm was stable in plasma at −86 °C and −20 °C but not at 4 °C, 22 °C or 37 °C over the tested period. Figs. 4 and 5 illustrates the stability for AQ and AQm in blood and plasma (3000 nM) using the ratio between AQ or AQm to CQ within each sample instead of the concentration. These figures conclude the result from Figs. 2 and 3 with one exception. AQ has to be considered unstable in plasma at 22 °C with only 82% recovered after 7 days versus 89% recovered with the traditional approach. It is quite clear when comparing the variation at stable temperatures in the figures (Figs. 4 and 5 versus Figs. 2 and 3) that the new approach significantly minimises the between-day variation. A statistical evaluation was performed using all the data at the temperatures where both AQ and AQm were considered stable with both approaches. Thus, using both the low (300 nM) and the high (3000 nM) concentration for AQ and AQm in blood at −86 °C, 4 °C and 22 °C and in plasma at −86 °C and −20 °C generated 78 observations with the traditional approach and 78

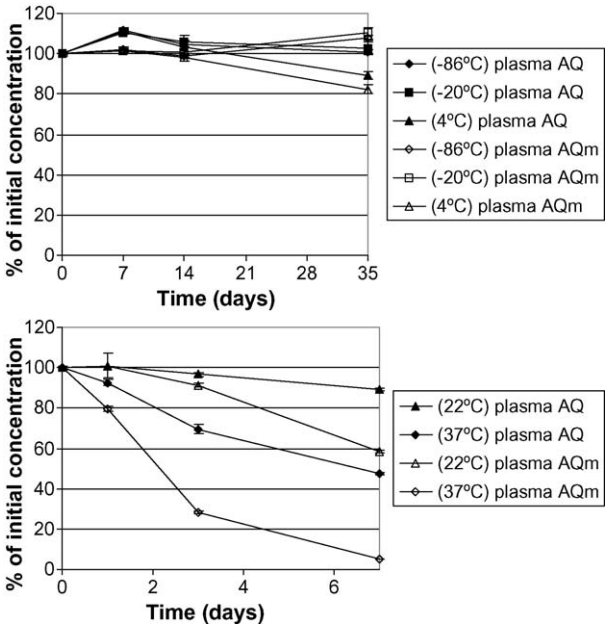

Fig. 3. Stability of AQ and AQm in plasma at different storage temperatures using freshly prepared calibration curves at each determination ($n = 3$, mean ± S.D.).

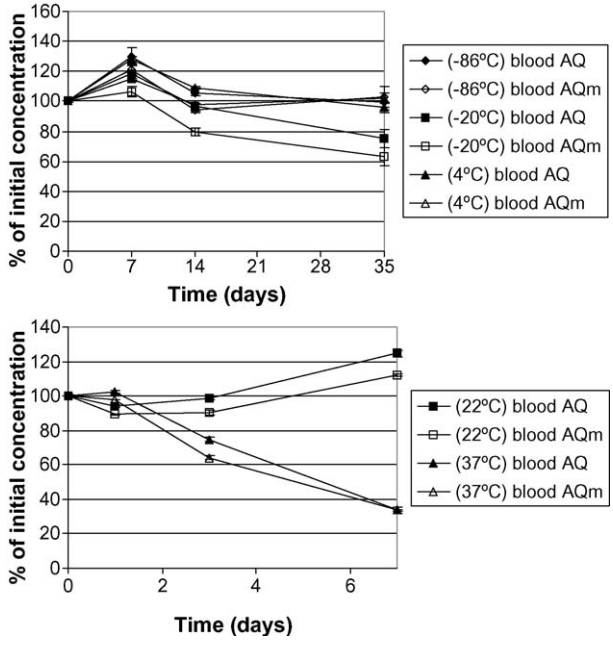

Fig. 2. Stability of AQ and AQm in whole blood at different storage temperatures using freshly prepared calibration curves at each determination ($n = 3$, mean ± S.D.).

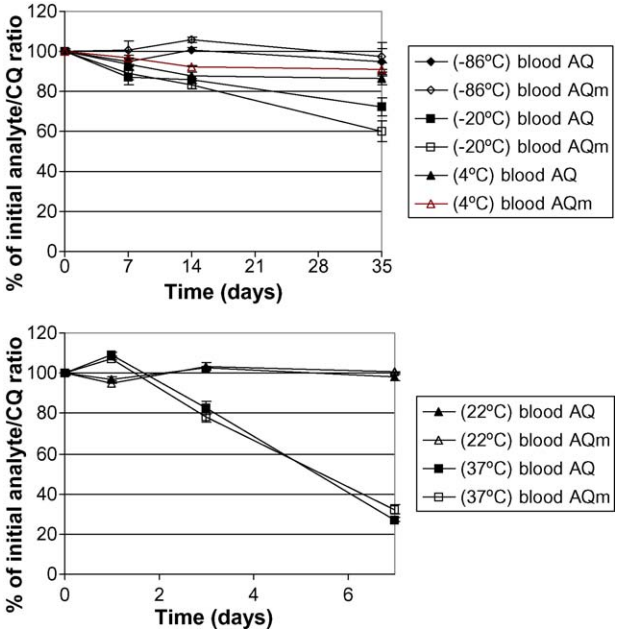

Fig. 4. Stability of AQ and AQm in whole blood at different storage temperatures using area ratio of AQ to CQ within sample ($n = 3$, mean ± S.D.).

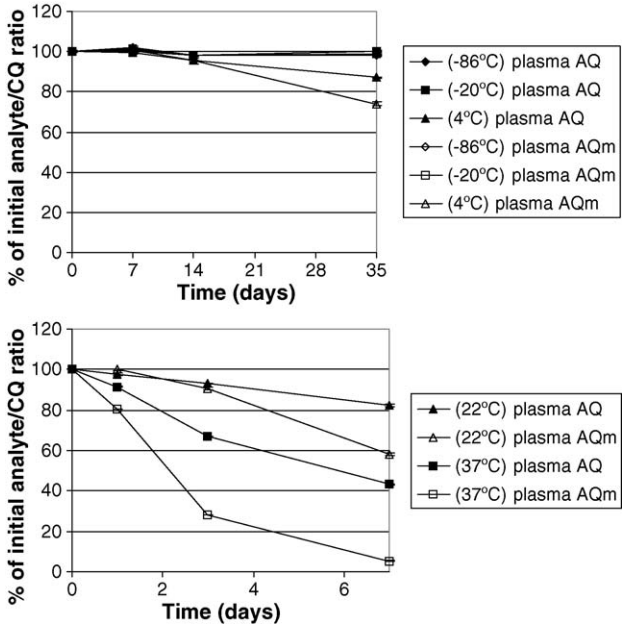

Fig. 5. Stability of AQ and AQm in plasma at different storage temperatures using area ratio of AQ to CQ within sample ($n = 3$, mean ± S.D.).

observations with the new approach. A *F*-test was used to test if there was a significant difference in variance between the two approaches. The *F*-test proved that the variance was significantly lower ($p < 0.001$) for the new approach ($s^2 = 16.6$) compared to the traditional approach ($s^2 = 96.7$). CQ was stable under all tested conditions in the present investigation as exemplified for CQ (300 nM) in plasma (Fig. 6), which is a necessity in order to use the ratio for evaluation. The stability of AQ and AQm at the low concentration (300 nM) gave comparable result as for the high concentration 3000 nM (i.e. stable/not stable at the same conditions).

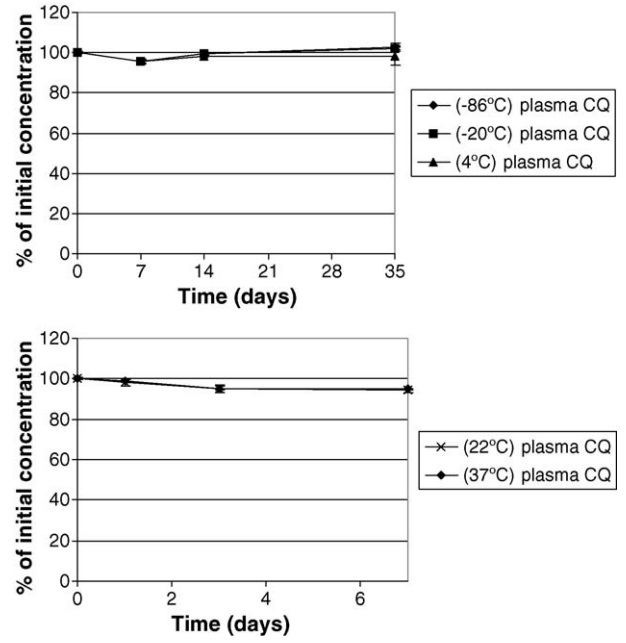

Fig. 6. Stability of CQ (300 nM) in plasma at different storage temperatures using freshly prepared calibration curves at each determination ($n = 3$, mean ± S.D.).

Both AQ and AQm were found to degrade in blood and plasma when stored at 37 °C. However, both have earlier been shown stable in dried blood spots at the same temperature for one month [8]. One important advantage of collecting biofluids onto sampling paper is the often-enhanced stability. These results suggest that collection of blood onto sampling paper should be considered when low temperature facilities are limited. Alternatively, different additives to stabilize AQ in blood or the possibility of immediate extraction and storage of extracted samples should be investigated. Minzi et al. tried to prevent possible oxidation of AQ by addition of sodium thiosulfate and ascorbic acid prior to drying it on paper [16]. No experiments to stabilize AQ in blood with these additives during the stability study were reported. It might be worthwhile to investigate whether an acidification of the blood would improve the stability of AQ. Sparidans et al. reported about significant improved storage stability for the nucleotide gemcitabine triphosphate in white blood cells (WBC) when stored with equal volume perchloric acid (0.8 M) [17]. Heinig et al. reported a similar phenomenon where the acidification of blood with phosphoric acid improved the stability for metrifonate and its metabolite in different biologic matrices [18].

The result in the present study further suggests a difference in stability for AQ/AQm in plasma versus blood at some temperatures. AQm was found stable in plasma but not in blood at −20 °C and stable in blood but not in plasma at 4 °C and 22 °C. These results were further confirmed by the analysis of triplicates in plasma and blood that had been stored at −86 °C and −20 °C for 148 days. Only 35% of initial AQ was recovered in the blood samples that had been stored at −20 °C while 97–99% were recovered in blood stored at −86 °C and plasma at both −86 °C and −20 °C. These results are likely to be explained by a protective accumulation of the drug within blood cells. Both AQ, AQm and CQ are lysosomotropic and accumulates by a pH gradient into the acidic lysosomes of the cells where they become protonated and trapped [19]. Both AQ and AQm accumulates in the white cells (especially lymphocytes) rather than in the red blood cells [19,20]. Thus, at moderate temperatures above 0, the stability is possibly enhanced in blood because AQ and AQm are protected within the white cells. At moderate low temperatures below 0, haemolysis caused by freezing instead enhances degradation, probably by the release of an unknown inducer from within the red blood cells. Noticeable was that blood aliquots that had been stored at −20 °C for 148 days were much darker than blood aliquots stored at −86 °C for the same amount of time. This could be an indication for oxygen release by haemoglobin in the samples. One option would then be that haemoglobin itself decomposes into monomers and finally free heme that theoretically could induce degradation of amodiaquine.

The variation between the different days was initially rather high (Figs. 2 and 3). The risk of making incorrect conclusions of trends is indeed greater if the samples are analysed only at two or three occasions. FDA guidelines recommendation that triplicates of two concentrations should be analysed at three separate

occasions might not be sufficient to make correct conclusions [7]. Hartmann et al. suggests that long-term stability is evaluated at three occasions but with at least six replicates at each occasion [21]. This seems like a sensible suggestion for bioanalytical methods where method precision (R.S.D.) often lays around 10% and the acceptance criteria 85% concentration ratio is used. We recommend that the approach proposed by Dadgar and Burnett should be used when the number of analysis occasions are few [6]. Simultaneous analysis of a compound that is already known to be stable can be used as a relative marker to ensure correct interpretation of results. It would in particular be feasible for methods using simple sample preparation techniques such as protein precipitation combined with LC–MS. Once a stable marker compound has been identified it can be used for several different analytes using gradient LC–MS in the stability study. It is important that the design and methodology is clearly described when results from stability studies are presented in bioanalytical validation articles.

4. Conclusion

A new approach using a stable marker within each sample for assessing stability has been proposed. The new approach significantly minimises day-to-day variation in long-term stability investigations and could be a useful additional tool to evaluate stability. A simple sample preparation (e.g. protein precipitation) combined with mass spectrometry could easily enable one single stability marker to be used for many different drugs. AQ and AQm were stable in plasma at −86 °C and −20 °C for 35 days, at 4 °C for 14 days and at 22 °C for 1 day. They were not stable at 37 °C after 1 day. AQ and AQm were stable in blood at −86 °C and 4 °C for 35 days, at −20 °C and 22 °C for 7 days and at 37 °C for 1 day. Blood samples containing AQ and AQm should be stored at −86 °C or colder as soon as possible after sampling.

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References

[1] C.S. Hatton, T.E. Peto, C. Bunch, G. Pasvol, S.J. Russell, C.R. Singer, G. Edwards, P. Winstanley, Lancet 1 (1986) 411–414.
[2] E.G. Rhodes, J. Ball, I.M. Franklin, Br. Med. J. (Clin. Res. Ed.) 292 (1986) 717–718.
[3] K.A. Neftel, W. Woodtly, M. Schmid, P.G. Frick, J. Fehr, Br. Med. J. (Clin. Res. Ed.) 292 (1986) 721–723.
[4] P. Olliaro, C. Nevill, J. LeBras, P. Ringwald, P. Mussano, P. Garner, P. Brasseur, Lancet 348 (1996) 1196–1201.
[5] V.G. Fowler Jr., M. Lemnge, S.G. Irare, E. Malecela, J. Mhina, S. Mtui, M. Mashaka, R. Mtoi, J. Trop. Med. Hyg. 96 (1993) 337–345.
[6] D. Dadgar, P.E. Burnett, J. Pharm. Biomed. Anal. 14 (1995) 23–31.
[7] Guidance for Industry Bioanalytical Method Validation, Department of Health and Human Services, Food and Drug Administration, Rockville, US, 2001.
[8] N. Lindegårdh, M. Forslund, M.D. Green, A. Kaneko, Y. Bergqvist, Chromatographia 55 (2002) 5–12.
[9] V.K. Dua, P.K. Kar, N.C. Gupta, V.P. Sharma, J. Pharm. Biomed. Anal. 21 (1999) 199–205.
[10] D. Dadgar, P.E. Burnett, M.G. Choc, K. Gallicano, J.W. Hooper, J. Pharm. Biomed. Anal. 13 (1995) 89–97.
[11] G.W. Mihaly, D.D. Nicholl, G. Edwards, S.A. Ward, M.L. Orme, D.A. Warrell, A.M. Breckenridge, J. Chromatogr. 337 (1985) 166–171.
[12] P. Winstanley, G. Edwards, M. Orme, A. Breckenridge, Br. J. Clin. Pharmacol. 23 (1987) 1–7.
[13] E. Pussard, F. Verdier, M.C. Blayo, J. Chromatogr. 374 (1986) 111–118.
[14] D.L. Mount, L.C. Patchen, P. Nguyen-Dinh, A.M. Barber, I.K. Schwartz, F.C. Churchill, J. Chromatogr. 383 (1986) 375–386.
[15] O.M. Minzi, M. Rais, J.O. Svensson, L.L. Gustafsson, O. Ericsson, J. Chromatogr. B 783 (2003) 473–480.
[16] O.M. Minzi, M. Rais, J.O. Svensson, L.L. Gustafsson, O. Ericsson, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 783 (2003) 473–480.
[17] R.W. Sparidans, M. Crul, J.H. Schellens, J.H. Beijnen, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 780 (2002) 423–430.
[18] R. Heinig, D. Zimmer, S. Yeh, G.J. Krol, J. Chromatogr. B Biomed. Sci. Appl. 741 (2000) 257–269.
[19] D.J. Naisbitt, J.E. Ruscoe, D. Williams, P.M. O'Neill, M. Pirmohamed, B.K. Park, J. Pharmacol. Exp. Ther. 280 (1997) 884–893.
[20] F. Laurent, S. Saivin, P. Chretien, J.F. Magnaval, F. Peyron, A. Sqalli, A.E. Tufenkji, Y. Coulais, H. Baba, G. Campistron, et al., Arzneimittelforschung 43 (1993) 612–616.
[21] C. Hartmann, J. Smeyers-Verbeke, D.L. Massart, R.D. McDowall, J. Pharm. Biomed. Anal. 17 (1998) 193–218.