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Rapid determination of tafenoquine in small volume human plasma samples by high-performance liquid chromatography–tandem mass spectrometry

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Abstract

A method was developed for the determination of tafenoquine (**I**) in human plasma using high-performance liquid chromatography–tandem mass spectrometry. Prior to analysis, the protein in plasma samples was precipitated with methanol containing [$^{2}H_{3}^{15}N$]tafenoquine (**II**) to act as an internal standard. The supernatant was injected onto a Genesis-C_{18} column without any further clean-up. The mass spectrometer was operated in the positive ion mode, employing a heat assisted nebulisation, electrospray interface. Ions were detected in multiple reaction monitoring mode. The assay required 50 μl of plasma and was precise and accurate within the range 2 to 500 ng/ml. The average within-run and between-run relative standard deviations were <7% at 2 ng/ml and greater concentrations. The average accuracy of validation standards was generally within ±4% of the nominal concentration. There was no evidence of instability of **I** in human plasma following three complete freeze–thaw cycles and samples can safely be stored for at least 8 months at approximately −70°C. The method was very robust and has been successfully applied to the analysis of clinical samples from patients and healthy volunteers dosed with **I**.

Keywords: Tafenoquine

1. Introduction

Tafenoquine (SB-252263), racemic 8-[(4-amino-1-methylbutyl)amino]-2,6-dimethoxy-4-methyl-5-(3-trifluoromethylphenoxy)quinoline succinate (**I**; Fig. 1), is currently under development by GlaxoSmithKline and the US army for prophylaxis and treatment of *Plasmodium falciparum* and *P. vivax* malaria. Compound **I** is an 8-aminoquinoline antimalarial agent with greater in vitro antimalarial activity than primaquine [1,2]. Compound **I** is effective against all stages of *Plasmodium* and is presently undergoing phase III clinical trials. It is metabolised slowly to a large number of very minor components.

Analytical methods have been reported for the measurement of **I** in plasma, which use high-performance liquid chromatography (HPLC) followed by either electrochemical detection [3] or fluorescence detection [4,5], but in the opinion of the authors none of these methods are suitable for high

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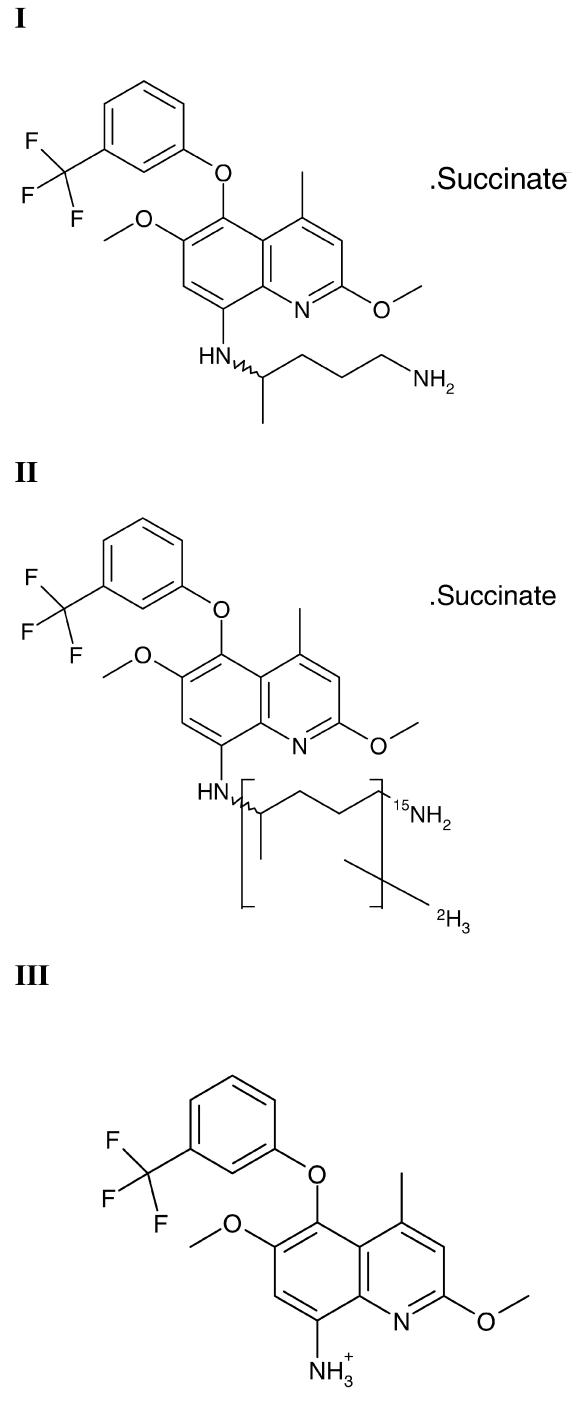

Fig. 1. Structures of tafenoquine **I**, [$^{2}H_{3}^{15}N$]tafenoquine (**II**, I.S.) and the major ion-radical observed in the product spectrum of both **I** and **II** at m/z 379 (**III**).

throughput support of clinical pharmacokinetic studies. In order to follow the pharmacokinetics of absorption and elimination during clinical efficacy trials, a high-performance liquid chromatography–tandem mass spectrometry (LC–MS–MS) method was developed for the determination of **I** in human plasma. This paper represents the first detailed description of that method. Measurement of the racemic **I** is justified because, as Karle and Olmeda have reported, there is negligible difference in the plasma concentrations of the two enantiomers, less than 10% in their study [3].

2. Experimental

2.1. Materials and reagents

All chemicals used were at least reagent grade and solvents of HPLC grade. They were purchased from Fisher Scientific (Loughborough, UK) and Romil (Cambridge, UK). Pure HPLC-grade water was obtained using an Elgastat MAXIMA (Elga, High Wycombe, UK) and gasses were high purity obtained from BOC (Luton, UK).

8-[(4-Amino-1-methylbutyl)amino]-2,6-dimethoxy-4-methyl-5-(3-trifluoromethylphenoxy)quinoline succinate (**I**; Fig. 1) and [$^{2}H_{3}^{15}N$]8-[(4-amino-1-methylbutyl)amino]-2,6-dimethoxy-4-methyl-5-(3-trifluoromethylphenoxy)quinoline succinate (**II**; Fig. 1) were synthesised by GlaxoSmithKline (PA, USA) and were greater than 98.6% pure as the salt form.

2.2. Preparation of drug standard solutions

I and **II** were stored away from light at all times and allowed to reach ambient temperatures before opening and weighing. Approximately 2 mg of **I** was weighed out and dissolved in methanol to give a solution containing 2 mg/ml **I**. The solution was stable for at least 1 week when stored at approximately 4 °C and protected from light. The 2 mg/ml stock solution was serially diluted with drug-free human plasma, to give working stock solutions containing 100, 2, 0.2, 0.02 and 0.002 μg/ml **I**. All working solutions were kept frozen at approximately −70 °C until required. Separate weighings were made for preparing the standard curves and for the estimation of precision.

Approximately 2 mg of **II** was weighed out and dissolved in methanol to give a solution containing 100 μg/ml **II**. This solution was protected from light and stored at approximately 4°C. The solution was stable for at least 1 week when stored under these conditions. On the day of assay this solution was diluted to 0.1 μg/ml with methanol, and used to precipitate plasma proteins in samples and standards.

2.3. Preparation of plasma standards and validation samples

Blood samples were taken from human subjects who had not received any medication in the previous 48 h, into EDTA tubes, mixed and centrifuged. The plasma was then frozen immediately and stored at approximately −70 °C until required for assay. Calibration standards were prepared over the range 2 to 500 ng/ml by adding appropriate amounts of **I** as a standard solution in plasma. Validation standards were prepared in bulk at concentrations of 2, 8, 200 and 500 ng/ml in plasma by adding appropriate amounts of **I**. Standards used for the estimation of precision were prepared in the same way as those used for calibration, but from a different stock solution.

2.4. Sample preparation

Samples and standards were prepared for injection onto the HPLC column after precipitation of the plasma proteins with methanol containing internal standard. Plasma (50 μl) and internal standard working solution (200 μl) were pipetted into a polypropylene tube (1.5 ml). The tube was capped and contents mixed on a vortex mixer for about 15 min using a multiple mixer. The sample was then centrifuged at approximately 14 000 *g* for 15 min. The supernatant was then carefully pipetted into a silanised 1.1-ml amber tapered autosampler vial. Finally, 4 μl of the extract was injected onto the HPLC column.

2.5. Instrumentation and chromatographic conditions

For this validation we used a Hewlett-Packard 1100 binary liquid chromatograph with integral column oven (Hewlett-Packard, Bracknell, UK) coupled to a PE Sciex API III plus tandem mass spectrometer (Perkin-Elmer Sciex, Ontario, Canada) using a heat assisted nebulisation, turbo-ionspray interface. A Genesis-C_{18} column (4 μm, 30×2.1 mm I.D., Jones Chromatography, Hengoed, Wales) was maintained at a constant 40 °C and the mobile phase [ammonium acetate buffer (1 m*M*, pH 2.5 with formic acid)–methanol (30:70)] flow-rate was 1.5 ml/min. The tandem mass spectrometer was operated in the positive ion mode with multiple reaction monitoring (MRM) and quantification was achieved by comparison of the chromatographic peak areas for tafenoquine (nominal positive ion 464 and nominal product ion 379) and internal standard (nominal positive ion 468 and nominal product ion 379). The major mass spectrometer operating parameters were typically: turbo gas flow=6.5 l/min; nebuliser gas pressure=$620 \cdot 10^3$ Pa; nebuliser gas flow=0.9 l/min; collision gas thickness=$300 \cdot 10^{13}$; dwell time=100 ms; pause time=5 ms; source delta pressure=1–1.5 inches of water (in. W.C.); turbo-ionspray temperature=600°C.

2.6. Method validation

A calibration curve containing eight points and a blank which was not included in the calculation was prepared in human plasma. **I** and **II** area ratios were determined and plotted against concentration of **I** to construct the curve. The response function was fitted as the simplest mathematical relationship for response versus concentration. The slope and intercept were determined by a weighted ($1/x^2$) least-squares linear regression. This weighting factor was chosen because it gave the best individual residual values.

Four pools of human plasma containing 2, 8, 200 and 500 ng/ml were prepared from a fresh stock solution of **I** as described above. Six replicate standards from each pool were extracted and analysed in each of three separate analytical runs. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. The within-run precision was estimated using the mean and the standard deviation of the six replicate results. The between-run precision was estimated by calculating the ratio of the standard deviation of the within-run means to the average of the within-run means, and expressed as a percentage. Accuracy was estimated as the ratio of the mean concentration by

analysis to the nominal concentration, and expressed as a percentage. Overall accuracy was reported as the average of the individual run accuracy estimations.

2.7. Stability

Two bulk standards at concentrations of 8 and 500 ng/ml in plasma were prepared in the same way as those used for the estimation of precision and accuracy (Section 2.3) and used for the estimation of freeze–thaw stability. Six replicate standards at each concentration were put through three complete freeze–thaw cycles, freezing to approximately −70°C, and analysed for **I** with six freshly prepared standards at each concentration prepared on the day of analysis.

Long-term stability was estimated using plasma samples from three healthy subjects following administration of **I** 400 mg daily for 3 days. The plasma samples from these subjects were assayed before and after storage at approximately −70 °C for a period of 8 months. The mean data before and after storage were compared to assess stability of the samples.

3. Results and discussion

3.1. Method development

Under the LC–MS–MS conditions described above the positive ion spectrum for **I** consisted predominately of a single ion at m/z 464, which corresponds to the protonated molecular (parent) ion. By selecting m/z 464, in the first mass analyser, and inducing fragmentation, the major ion observed in the product spectrum was detected at m/z 379. This ion (**III**; Fig. 1) gave a signal intensity greater than any other ion observed in the spectrum and was therefore selected for sensitive quantification of **I**. This fragment is consistent with the loss of a pentamine moiety. The positive ion and product spectra for **II** were very similar to those for **I** except that the parent ion at m/z 468 (**III**; Fig. 1) shows the presence of the stable labels, and the major fragment ion at m/z 379 shows the loss of the pentamine which contained the stable labels.

Since the method was required to support clinical pharmacokinetic studies with **I**, an internal company requirement was that the assay had to be precise to a relative standard deviation (RSD) within 15% throughout the required concentration range. Accuracy (±15% bias) and linearity had also to be demonstrated throughout the required range. In addition, the method had to have a high rate of sample throughput and be rugged enough to be able to support a large and very intensive development programme. These conditions were met because of the simple approach to sample preparation that yielded an extract which was amenable to subsequent quantitative LC–MS–MS analysis.

3.2. Separation and specificity

No significant interfering peaks that co-eluted with **I** or **II** were observed. These results were good evidence that the assay was selective for **I** over endogenous compounds. Compounds **I** and **II** both eluted together with a retention time of under 1 min. No known metabolites have been observed to interfere with this assay. A typical chromatogram of human control plasma with **I** added at the lower limit of quantification (LLQ; 2 ng/ml) and containing **II** is shown in (Fig. 2).

3.3. Linearity, precision and accuracy

The mean back-calculated concentrations for three calibration curves, one for each of three analytical runs, were within 3% of the nominal concentration. Linearity was demonstrated over the range 2–500 ng/ml for tafenoquine extracted from 50 μl of plasma. This is an appropriate range over which to study the pharmacokinetics of **I** following administration of **I** 25 mg to 400 mg daily for 3 days to healthy subjects and to study the pharmacokinetics of **I** in patients (Fig. 3). Concentrations of **I** in plasma which were greater than 500 ng/ml were measured after dilution of the sample with drug-free human plasma.

The within-run and between-run precision values for the assay were better than 7% RSD at all concentrations studied (Table 1). Mean accuracy (calculated as the mean over three runs of the individual run mean concentration by analysis, to

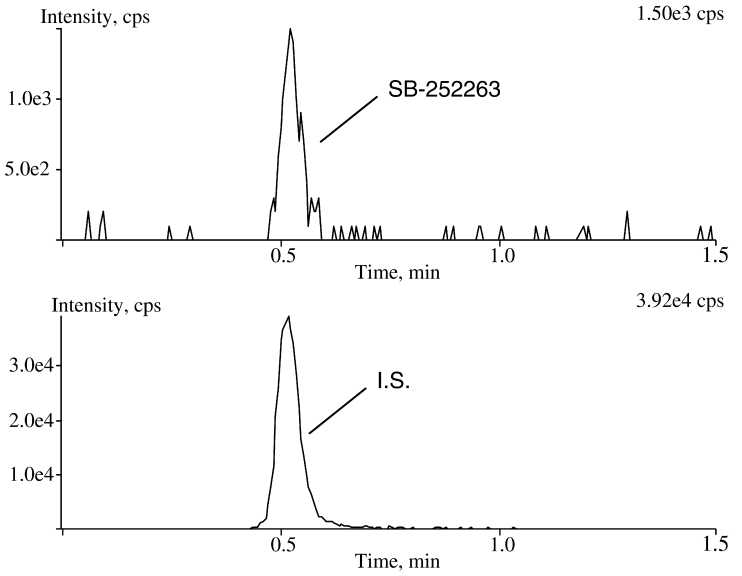

Fig. 2. Representative MRM chromatogram of **I** (SB-252263) in human plasma at the LLQ of the assay (2 ng/ml).

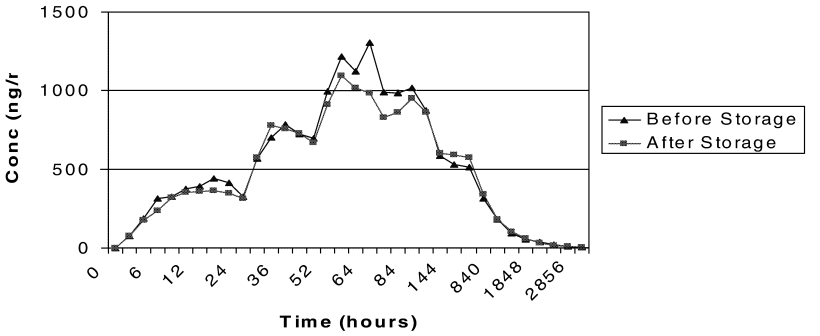

Fig. 3. Plasma concentrations of **I** in healthy human subjects following administration of **I** 400 mg daily for 3 days, measured (a) before and (b) after storage at −70 °C for 8 months.

nominal concentration, and expressed as a percentage) was generally within ±4% of nominal at the concentrations studied (Table 1). The method was therefore shown to be sufficiently accurate and precise to support clinical pharmacokinetic studies with **I**.

Table 1
Precision and bias for **I** in human plasma

	Nominal **I** (ng/ml)			
	2	8	200	500
Average within-run precision (%)	6.46	1.98	1.16	1.66
Between-run precision (%)	4.38	7.24	0.55	4.74
Average bias (%)	−3.69	3.45	−1.84	−0.36

3.4. Stability

Results obtained for six replicate validation samples containing **I** at each of two concentrations (8 and 500 ng/ml) which were put through three complete freeze–thaw cycles, freezing to approximately −70 °C and those for six replicate samples prepared freshly at the same concentrations differed by less than 9%. Hence there was no evidence of instability of **I** in human plasma following three complete freeze–thaw cycles.

The analysis of post-dose samples stored at approximately −70 °C for up to 8 months showed no significant deterioration in the performance of the assay in that plasma concentration time profiles before and after storage were similar, (mean of the % difference at each time point on the profile was −4.2 with a range of −24.5 to 11.5; Fig. 3). Hence there was no evidence of instability of **I** in human plasma when stored at approximately −70 °C for 8 months. It has also been previously reported that **I** is stable in plasma for at least 134 days at approximately −20 °C [6].

4. Conclusion

An accurate and precise LC–MS–MS assay using protein precipitation sample pretreatment was developed for the assay of **I** in a small volume of human plasma, which consists of the precipitation of protein in plasma with methanol and injection of the centrifuged supernatant for isocratic reversed-phase chromatography with MS–MS detection. Consequently the analytical procedure was simple and the method had a high sample throughput. This uncomplicated approach was possible because of the selectivity of LC–MS–MS which allowed accurate measurement with minimal sample clean-up. The method has proven to be very robust and has been successfully applied to the analysis of clinical samples from patients and healthy volunteers dosed with **I**.

The method is accurate and precise at levels as low as 2 ng/ml and has a range up to 500 ng/ml which is adequate for most clinical studies with **I**. Higher concentrations can be measured, if required, after first diluting the sample with drug-free human plasma. There was no evidence of instability of **I** in human plasma following three complete freeze–thaw cycles and samples can safely be stored for 8 months at approximately −70 °C or for at least 134 days at approximately −20 °C.

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