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Note

Improved high-performance liquid chromatographic method for the determination of quinine in plasma

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Quinine has been used as an anti-malarial drug since 1633, although its use declined following the introduction of chloroquine in 1934. However, due to the emergence of strains of *Plasmodium falciparum* malaria resistant to chloroquine and other anti-malarial drugs there has been renewed interest in quinine.

Non-specific colorimetric and fluorometric methods have been used to quantitate quinine and its metabolites in biological fluids [1–3]. Recently, more specific high-performance liquid chromatographic (HPLC) techniques have been developed using complicated extraction procedures and/or reversed-phase, ion-pair chromatography [4–7]. These methods are not suitable for single-dose pharmacokinetic studies because they lack sensivity and have poor specificity. While these methods are potentially specific for quinine, metabolite interference in plasma was not examined. Mihaly et al. [8] described a method utilizing HPLC which involves a time-consuming extraction procedure.

The present report describes an HPLC assay whose specificity for quinine has been examined. The method is a reversed-phase HPLC assay using a nitrile column and fluorescence detection. The procedure results in the elution of quinine as a sharp peak and has sufficient sensitivity (50 ng/ml) for single-dose pharmacokinetic studies. The specificity of the assay was assessed by extracting the major metabolites of quinine from the urine of volunteers who had received a single 300-mg oral dose of quinine and then isolating the metabolites using two-dimensional thin-layer chromatography (TLC). The method separates quinine

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from its major metabolites and endogenous compounds in plasma. Further, the inadequate specificity of a non-chromatographic fluorometric method has been confirmed.

EXPERIMENTAL

Chemicals and reagents

Acetone and ethanol (AR grade) were obtained from May & Baker (Melbourne, Australia). Acetonitrile and methanol (Chromar) were obtained from Mallinkrodt (Sydney, Australia). Ammonium hydroxide, dipotassium hydrogenphosphate trihydrate and quinine sulphate were supplied by British Drug Houses (Sydney, Australia). Toluene, sulphuric acid and isoamyl alcohol were obtained from Ajax Chemicals (Sydney, Australia). Diethyl ether (Mallinkrodt) was distilled before use. Stock solutions of quinine sulphate were prepared by dissolving the compound in deionized distilled water. Quantitation curves were constructed by spiking drug-free plasma with an aqueous solution of quinine sulphate to produce standards equivalent to 0.1, 0.5, 1.0, 2.5 and 5 μg/ml quinine. Aliquots of the standards (0.5 ml) were frozen and remained stable for at least one month.

Apparatus and chromatographic conditions

HPLC assays were carried out on a Varian 5020 liquid chromatograph (Varian, Sydney, Australia). Samples were injected with an ICI Kortec K65 autosampler (ICI, Sydney, Australia), and an Hitachi F1000 fluorometer (Hitachi Naka Works, Hitachi, Tokyo, Japan) was used as the detector with excitation set at 350 nm and emission at 450 nm. Detector output was quantified using a Hewlett-Packard integrator (Model 3390A, Hewlett-Packard Australia). Separations were performed on a 5-μm nitrile column (25 cm $\times$ 4.6 mm I.D., Regis, Morton Grove, IL, U.S.A.).

The compounds were eluted with a mobile phase of acetonitrile–methanol–phosphate buffer (6:2:2, v/v/v). The phosphate buffer was a 0.01 *M* solution of dipotassium hydrogenphosphate trihydrate adjusted to pH 3.7 with orthophosphoric acid. The column temperature was 24°C and the flow-rate was 1.0 ml/min.

Extraction procedures

Plasma was extracted using the method of Edstein et al. [4]. Plasma (0.5 ml) was placed in a 5-ml glass screw-capped tube containing acetonitrile (1.0 ml). After thorough mixing on a Vortex the sample was centrifuged at 9000 *g* (5 min, 15°C) and 100 μl of the supernatant were injected into the HPLC system.

Isolation of metabolites from urine

Quinine and its metabolites were extracted from urine (2 ml) into diethyl ether (8 ml) after the addition of 1 *M* sodium hydroxide (400 μl). The mixture was mechanically shaken for 10 min and then centrifuged (1100 *g*, 10 min). The aqueous phase was discarded and the organic phase evaporated to dryness. The residue was resuspended in acetone (100 μl) and applied to an acetone-washed

TLC plate (silica gel 60 F_{254}, 0.25 mm thickness, E. Merck, Darmstadt, F.R.G.). The plate was developed in a mobile phase of 0.75% ammonium hydroxide in methanol, dried and redeveloped in the same solvent. The plate was rotated through 90° and developed in a second mobile phase of methanol–acetone (4:1, v/v) [2]. The fluorescent areas corresponding to quinine and its metabolites, were removed from the plate and the compounds eluted into 8 ml of ethanol–acetone (1:1, v/v) [2]. After evaporating the organic solvent to dryness the residue was resuspended in acetonitrile and injected into the HPLC system.

Spectrofluorometric method

A modified extraction similar to that of Cramer and Isaksson [1] was used to extract quinine from plasma. Plasma (0.5 ml) was diluted with doubly distilled water (0.5 ml) and made alkaline with 0.1 *M* sodium hydroxide (1 ml). The sample was extracted for 10 min with 0.5% isoamyl alcohol in toluene (10 ml) and centrifuged at 1100 *g* for 10 min. The organic phase was extracted for 10 min with 0.1 *M* sulphuric acid (5 ml) and centrifuged at 1100 *g* for 10 min. The organic layer was discarded and the quinine in the aqueous phase was quantitated spectrofluorometrically in a Perkin-Elmer fluorescence MPF-3 spectrophotometer (Perkin-Elmer, Sydney, Australia) with excitation at 350 nm and emission at 450 nm.

RESULTS AND DISCUSSION

Reversed-phase HPLC on a nitrile column with fluorescence detection was an effective method of quantifying quinine in human plasma. Typical chromatograms obtained from blank plasma, plasma containing quinine standard and plasma taken 4 h after the administration of quinine to a volunteer are shown in Fig. 1. Interference by endogenous substances did not occur in drug-free plasma (Fig. 1a). Two quinine metabolites and a contaminant of quinine, isolated from the urine of a volunteer who had received a 300-mg single dose of quinine, were identified using gas chromatography–mass spectroscopy. Under the conditions described, the metabolites had elution times of 6.78 min (hydroxyquinine) and 7.01 min (quinine epoxide) which did not interfere with quinine (7.7 min). The contaminant, dihydroquinine, co-chromatographed with quinine at comparative concentrations of less than 3%.

The standard curves for quinine were linear over the concentration range 0.1–5.0 μg/ml. The correlation coefficient for the standard curves over this concentration range was >0.998. The coefficient of variation was $<10\%$ for all concentrations measured (Table I). The sensitivity of the method was approximately 50 ng/ml of plasma and the extraction recovery was $99.8 \pm 2.7\%$ (mean ± S.D.) (Table I).

A comparison was made between a non-chromatographic fluorometric method [4] and the HPLC technique described, by assaying plasma from two fasting subjects 0–17 h after taking 300 mg of quinine sulphate. The fluorometric technique yielded consistently higher values than the HPLC method with the difference being as high as 35% in one subject. These data were statistically different

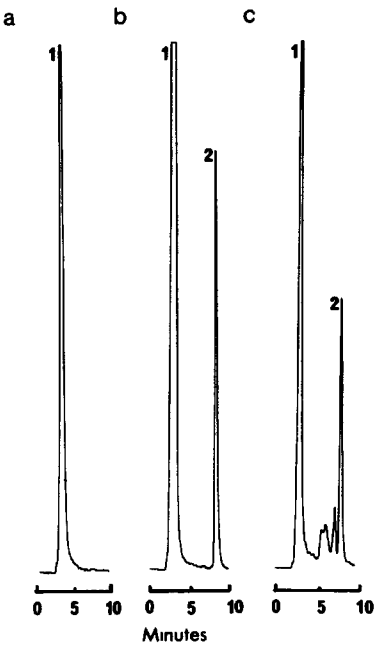

Fig. 1. Chromatograms of plasma extracts. (a) Blank human plasma; (b) human plasma containing 5 μg/ml quinine; (c) plasma collected 4 h after 300 mg quinine were administered orally to a subject. Peaks: 1 = solvent front; 2 = quinine.

TABLE I

REPRODUCIBILITY AND RECOVERY OF STANDARDS EXTRACTED FROM HUMAN PLASMA ($n=7$)

Concentration (μg/ml)	Coefficient of variation (%)	Recovery (%)
5.0	6	98
2.5	5	104
1.0	6	99
0.5	5	101
0.1	10	97

($p<0.009$). This would suggest that the fluorometric technique is not specific and detects both quinine and its metabolites.

The specific and sensitive method described here for the determination of quinine in human plasma employs HPLC with fluorescence detection. Sample preparation is a simple protein precipitation followed by HPLC analysis of the supernatant on a reversible nitrile column. The method is characterized by high recovery, good reproducibility and it is well suited for single- and multiple-dose pharmacokinetic studies in humans.

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