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Letter to the Editor

Removal of an endogenous fluorescent compound from urine to allow quantitation of low concentrations of hydroxychloroquine and metabolites by high-performance liquid chromatography

Sir,

An assay has been developed to quantitate hydroxychloroquine (HCQ) and its metabolites in plasma and whole blood [1]. Using this procedure for urine analysis the limit of detection was unsatisfactory for accurate quantitation of low concentrations (less than about 50 ng/ml). An endogenous fluorescent compound, extracted from alkalinised urine by the single ether extraction used in the assay [1], obscured peaks in the urine chromatogram when using high sensitivity of detection.

The method described here removes this interfering peak by first adding a reducing agent to the urine, then extracting the acidified urine with chloroform. The chloroform phase is discarded and the compounds are extracted from the aqueous phase by a single ether extraction, as previously described for blood and plasma [1]. The high-performance liquid chromatographic (HPLC) equipment and the assay conditions used were as previously described [1].

A drug-free urine sample was alkalinised and extracted once with diethyl ether, as described for the analysis of plasma and blood [1]. After injection of the reconstituted extract into the HPLC system, the first 3 min of eluent from the detector was collected. A mass spectrum of this eluent was obtained on a Finnigan 3200 gas chromatograph—mass spectrometer.

Numerous high-molecular-weight fragments (m/z 500—600) appeared in the mass spectrum obtained of the interfering peak from the blank urine. This indicated that the peak did not contain mainly low-molecular-weight drugs such as caffeine or nicotine, or phthalates from plastics or rubber. Comparison with reference spectra of these compounds confirmed this. This suggested that endogenous compounds, such as bilirubin conjugates and/or breakdown products, which have molecular weights in this region and also fluoresce, could be primarily responsible for the interference.

The method developed for removing the interfering peak was an adaptation of a method for quantitation of urobilinogen [2], discarding the organic phase containing the contaminants, rather than assaying it.

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Urine (1 ml), with chloroquine added as the internal standard, was mixed with 0.5 ml of aqueous ferrous sulphate solution (200 g/l $FeSO_4 \cdot 7H_2O$) and 0.5 ml of aqueous sodium hydroxide solution (100 g/l), and left to stand in the dark for 1 h. The mixture was then centrifuged at 1200 *g* for 5 min, and the supernatant decanted into a tube containing 20 mg ascorbic acid. Acetic acid solution (5 ml; 25 ml/l glacial acetic acid) was added, and the solution vortexed for 1 min with 5 ml chloroform. The mixture was centrifuged at 1200 *g* for 5 min, then the aqueous phase transferred to another tube using a silanized pipette. The chloroform was discarded. Ammonia solution (2 ml; 30%) was added to the aqueous phase, then the solution vortexed for 1 min with 10 ml of diethyl ether. The phases were separated by centrifuging for 5 min at 1200 *g*. The aqueous phase was frozen by immersion of the tube in a slurry of dry ice and acetone and the ether phase decanted and evaporated to dryness under a stream of nitrogen at room temperature. The extract was reconstituted in 100 μl of acetonitrile—0.1 *M* sulphuric acid (1:1) and 20 μl were injected into the HPLC system.

As HCQ and metabolites are basic compounds they are fully ionised at low pH and not extracted into chloroform. Table I shows the recoveries from urine of the compounds at low concentrations, with the preliminary reduction and chloroform extraction steps, and also recoveries of the compounds from urine at higher concentrations, using only the single ether extraction. The recoveries are similar indicating no major loss of compounds in the preliminary steps. The precision of the method at low and high drug concentrations, expressed as relative standard deviations, is also shown in Table I.

The method developed here was not concerned with identifying the interfering compound(s). Removing the interfering fluorescence to allow more sensitive determination of HCQ and metabolites in urine was the aim. This was achieved and the interfering peak in the chromatogram reduced to insignificant levels.

TABLE I

RECOVERIES FROM URINE AND RELATIVE STANDARD DEVIATIONS (R.S.D., $n = 3$) OF HYDROXYCHLOROQUINE AND METABOLITES

(A) High concentrations using a single ether extraction; (B) low concentrations using reduction followed by single ether extractions.

Compound	A				B			
	Recovery (%)		R.S.D. (%)		Recovery (%)		R.S.D. (%)	
	100 ng/ml	400 ng/ml	100 ng/ml	400 ng/ml	5 ng/ml	10 ng/ml	1 ng/ml	10 ng/ml
Hydroxychloroquine	92	92	5	1	85	90	11	3
Desethylhydroxy-chloroquine	73	74	2	3	54	58	8	1
Desethylchloroquine	88	83	1	1	80	80	1	2
Bisdesethyl-chloroquine	62	61	2	2	50	55	11	12

It is envisaged that this procedure may enhance the sensitivity of other assays in urine using fluorescence detection for basic compounds resistant to the reduction process.

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