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Note

Measurement of 4-hydroxyanisole in serum by direct injection high-performance liquid chromatography

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Monitoring concentrations of potentially toxic drugs in biological fluids is essential in balancing therapeutic benefit against adverse effects of the treatment Study of pharmacokinetics allows prediction of the most appropriate dose and mode of administration of the particular drug. A prerequisite for such investigations is an assay for the drug in question.

Intra-arterial administration of 4-hydroxyanisole (4HA) has been shown to be effective in the treatment of recurrent malignant melanoma [1,2]. *In vitro* studies using a melanoma cell line [3] have been used to suggest an appropriate therapeutic dose, however, there are very few studies on the serum drug levels attained using different treatment regimes in patients with malignant melanoma [4].

Measurement of 4HA in plasma by HPLC (using a 25-cm Hypersil ODS column) after prior treatment with acetonitrile, to precipitate plasma proteins and to extract the drug, has been reported [5]. In this study, we describe a direct injection high-performance liquid chromatographic (HPLC) method for assay of 4HA in serum using a Pinkerton internal surface reversed-phase (ISRP) column. Using plasma samples spiked with a variety of compounds, Hagerstrom and Pinkerton [6] demonstrated that Pinkerton ISRP columns are suitable for assay of drugs. Such columns have been used to develop methods for the measurement in patients' samples of paracetamol and salicylate [7], theophylline and caffeine [8] and anti-epileptic drugs [9].

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EXPERIMENTAL

Reagents

The methanol used in the preparation of the mobile phase was HPLC grade (BDH, Poole, U.K.). The internal standard, 4-ethoxyphenol, was purchased from Sigma (Poole, U.K.) and the 4-hydroxyanisole (4-methoxyphenol) was obtained from Aldrich via the Hospital Pharmacy. The same supply of 4HA was used for preparation of the intra-arterial infusion.

Apparatus

A 150 mm × 4.6 mm I.D. Pinkerton ISRP column (5-μm particles) and a pre-column (Regius Labs., Chicago, IL, U.S.A.) were used at ambient room temperature with a Philips (Cambridge, U.K.) PU4015 pump, an LC–UV detector, a PU 4811 computing integrator and a Spark Holland (Beckman) autoinjector with a 20-μl injection loop. The column and pre-columns were stored in 100% acetonitrile. The pre-columns were changed after approximately 400 injections. The performance characteristics of the 15-cm column did not change significantly after about 2000 injections but, eventually, the pressure increased and elution profiles changed, possibly due to retention on the column of lipid and some protein material We have yet to find a satisfactory way of regenerating the column at this stage.

Procedure

Samples were obtained from patients undergoing treatment with 4HA (10 g in 500 ml of saline infused over a 30-min period). Serum samples were frozen until required. Standard (100 μl at concentrations of 0–400 mg/l in serum or mobile phase for linearity check and routinely 80 mg/l in mobile phase) or serum (100 μl) were mixed with 1 ml of internal standard (20 mg/l 4-ethoxyphenol in mobile phase), and 20 μl of the mixture were injected on to the column. The mobile phase (0.1 mol/l potassium phosphate buffer pH 6.8 containing 2.5% methanol) was pumped at 1.2 ml/min and the output was monitored at 280 nm with a full scale absorbance deflection of 0.16. Peak-height ratios were used for the calculation of results.

RESULTS AND DISCUSSION

The chromatographic conditions described resulted in good separation of 4HA and internal standard with retention times of about 5 and 8 min, respectively, both compounds eluting well after the proteins which come straight through the column (Fig. 1). Calibration curves for both aqueous and serum-based standards were performed three times with concentrations of 4HA between 400 and 1.56 mg/l in nine doubling dilutions. Aqueous (Fig. 1a) and serum-based standards gave linear and coincident calibration curves up to a concentration of

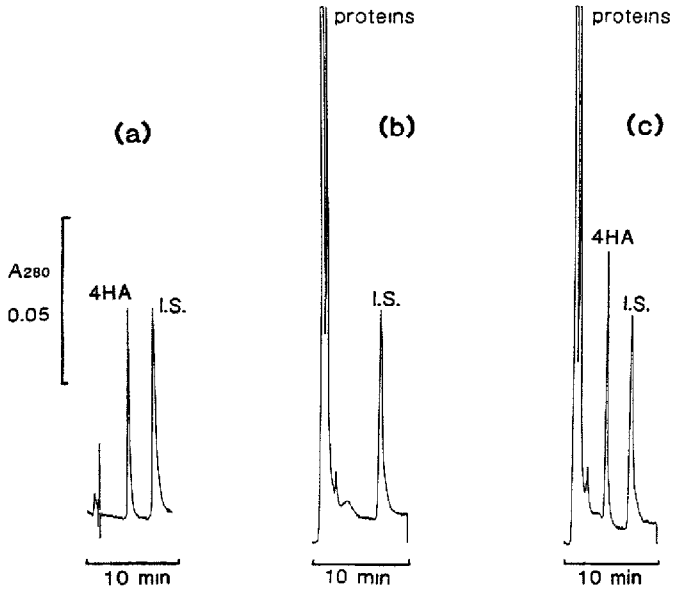

Fig. 1 Chromatograms of 4HA and internal standard (I.S.) in (a) aqueous standard (80 mg/l), (b) serum from a patient before therapy (t = 0 min) and (c) serum from the same patient 5 min after the end of the infusion of 10 g over 30 min

at least 400 mg/l ($y = 0.0144x + 0.005$; y is peak-height ratio and x is concentration; $r = 0.9999$), indicating full recovery of 4HA from serum. It can, therefore, be assumed that the method measures total concentrations of 4HA in serum The minimum level to be integrated as a peak under the conditions described was 2 mg/l. Sera from patients before infusion of 4HA (t = 0 min) were chromatographed with (Fig. 1b) and without internal standard to check for the presence of interfering endogenous peaks; no extra peaks were seen in the nine patients studied so far. After 4HA infusion, no metabolites were detected, only the parent compound (Fig. 1c; t = 5 min). Peak levels of 4HA in patients after infusion of 10 g over 30 min were between 80 and 140 mg/l.

The assay performance was assessed on patients' samples. The within-batch coefficient of variation for the method was 2.2% (ten determinations of the same serum; mean = 62 mg/l) and the between-batch coefficient of variation was 4.9% (a sample determined on ten different days; mean = 47 mg/l).

Table I shows retention indices for several drugs, none of which interfered in the assay of 4HA, all except codeine eluting before the 4HA peak. The list of drugs tested is not exhaustive since treatment regimes with 4HA allow a check to be made on possible interference from prescribed medication. Severe haemolysis and lipaemia had no effect on the measurement of 4HA in spiked serum. The method works equally well on plasma samples and may be used on saponin-haemolysed whole blood [10].

TABLE I

RETENTION INDICES (R_t DRUG/R_t INTERNAL STANDARD) OF SOME DRUGS

Drug	Retention index
4HA	0.65
Paracetamol	0 36
Salicylate	0.40
Metoclopramide	0 48
Caffeine	0 48
Theophylline	0.50
Codeine	–

This direct injection method provides a rapid, interference-free assay for the measurement of 4HA in serum or plasma from patients undergoing treatment to allow pharmacokinetic studies and the evaluation of different treatment regimes.

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