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DETERMINATION OF THE ANTIOXIDANT 3-*TERT*.-BUTYL-4-HYDROXYANISOLE IN RAT PLASMA USING HIGH-RESOLUTION GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method is described for the determination of the antioxidant 3-*tert*.-butyl-4-hydroxyanisole in rat plasma using high-resolution capillary gas chromatography—mass spectrometry with selective ion monitoring. Following the addition of the isomer 2-*tert*.-butyl-4-hydroxyanisole, used as an internal standard, extraction was made with *n*-hexane and the extract derivatized with heptafluorobutyric anhydride.

The gas chromatographic separation was carried out on a SE-52 fused silica capillary column and the derivatized 3-*tert*.-butyl-4-hydroxyanisole and its isomer detected by recording the intensities of their common fragment ion at *m/e* 361. The sensitivity of the method allowed the antioxidant to be measured in 0.1-ml rat plasma samples down to a level of 10 ng/ml with a high degree of specificity and accuracy. The method has been applied to a preliminary pharmacokinetic study in rats after oral dosage.

INTRODUCTION

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used as antioxidant food additives.

BHA and BHT have been shown to be powerful perturbing agents for biomembranes in vitro [1]. BHA exhibits a very low toxicity in mammals when given orally, while its toxicity in the rat is greatly enhanced after intraperitoneal administration [2]. The observation that BHA is a substrate for mam-

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malian intestinal peroxidase would suggest that peroxidative oxidation at the intestinal wall may represent a contribution to the inactivation of BHA and other phenol derivatives potentially toxic to mammals [3].

In spite of lacking evidence of hazard from BHA at current use levels, the Federation of American Societies for Experimental Biology committee on GRAS (generally recognised as safe) substances has suggested the necessity to give an "interim status" to BHA until additional studies on the toxicity and metabolism of this compound in various animal species resolve the existing uncertainties [4].

As a contribution to a better knowledge of the toxicity and metabolism of BHA, a study on the kinetic behaviour of this compound in the rat has been started.

For the purpose of this study an assay procedure was required which would allow BHA to be measured in small volumes (0.1 ml) of rat plasma with a high degree of specificity and sensitivity. A number of methods using gas chromatography (GC) have previously been developed for detecting and quantifying this antioxidant in food products (references to these methods are given in a recent review article [5]). A spectrophotometric method suitable for aqueous samples has also recently been reported [6]. This assay procedure however requires 4 ml of aqueous sample and, as admitted by the authors, does not have the necessary specificity required for low-level determinations in biological samples. El-Rashidy and Niazi [7] have described a GC method using a packed column with flame ionization detection for determining BHA in human plasma and urine. The detection limit was reported as less than 100 ng/ml sample but the method required 1 ml plasma or 4 ml urine. In order to lower the detection limit and improve assay specificity we have developed a method for determining BHA in rat plasma based on the use of high-resolution capillary GC combined with mass spectrometry (MS).

MATERIALS AND METHODS

3-*tert.*-Butyl-4-hydroxyanisole (3-BHA) was a Fluka Chemical obtained from Prokeme (Florence, Italy). 2-*tert.*-Butyl-4-hydroxyanisole (2-BHA) was prepared from a commercial sample of 3-BHA from Sigma (London) (Poole, Great Britain) which contained approximately 7% of the 2-isomer. The separation of the isomers was carried out on a Sephadex LH-20 column using the solvent mixture chloroform—cyclohexane (1:1, v/v) according to the procedure described by Kauffman [8]. The purity of the isolated 2-BHA was confirmed by thin-layer chromatography, GC and GC—MS.

Heptafluorobutyric anhydride supplied by Pierce and Wariner (Chester, Great Britain) was stored at 0°C in dark bottles. *n*-Hexane and pyridine obtained from BDH (Poole, Great Britain) were of AnalaR grade and were used without further purification. Ethyl acetate (Fisons, Loughborough, Great Britain) of Distol Reagent grade was dried by adsorptive filtration through Alumina Woelm act. 1 (Koch-Light, Colnbrook, Great Britain).

Animal studies

Male LAC:Porton rats (200—230 g) were anaesthetised with diethyl ether

and a PE50 cannula inserted into the ventral tail artery. The animals were put into restraining cages and allowed to recover for 1 h before oral administration of 1 ml/kg of a 200 mg/ml solution of 3-BHA in dimethyl sulphoxide. Blood samples (200—300 μl) were withdrawn from the cannula into heparinised tapered tubes at 10, 20, 30 min and 1, 2, 3, 4, 5 and 24 h after administration of 3-BHA. Samples were immediately centrifuged at 2000 *g* for 5 min and the plasma fractions stored frozen until required for analysis.

Extraction and derivatization

All glassware was cleaned and silanized as described previously [9]. A 0.1-ml aliquot of rat plasma was diluted to 0.5 ml with distilled water in a 30-ml glass stoppered centrifuge tube and 20 μl of the internal standard in ethyl acetate containing either 40 ng or 4 ng 2-BHA were added. The sample was extracted with 3 ml *n*-hexane by Vortex mixing for 30 sec followed by centrifugation at 1500 *g* for 2 min. The organic layer was transferred to an 8-ml glass tube fitted with a Teflon®-lined screw cap. The plasma was extracted with a further 1 ml *n*-hexane and the combined extracts evaporated to dryness under nitrogen at 0°C. The dried extract was derivatized by adding 500 μl hexane, 20 μl pyridine and 25 μl heptafluorobutyric anhydride and reacting for 30 min at 60°C. After cooling 2.0 ml *n*-hexane and 1.0 ml 0.1 *M* phosphate buffer (pH 6.9) were added and the mixture shaken on the Vortex mixer for 30 sec and then centrifuged at 1500 *g* for 2 min. The hexane layer was transferred to a 3-ml Reacti-Vial (Pierce and Warriner) and the extract evaporated under nitrogen at 0°C before finally dissolving in ethyl acetate for GC—MS analysis.

Gas chromatography—mass spectrometry

The instrument used in this study was a 70-70F VG Micromass double focussing mass spectrometer linked with a VG 2035 Data System. The GC separations were made on a 25 m × 0.25 mm I.D. fused silica capillary column (Phase Separations, Queensferry, Great Britain) coated in the authors' laboratory with SE-52 stationary phase by the static coating procedure to give a film thickness of ca 0.3 μm. The capillary column was installed in a Pye-Unicam Series 204 gas chromatograph interfaced to the mass spectrometer with glass-lined stainless-steel tubing. The outlet end of the fused silica capillary was fed through the GC—MS interface up to the ion source entry tube. The capillary column connections in the GC oven were made using graphite ferrules. Samples were introduced into the capillary using either a falling needle solid injector or a solvent splitting device (10:1 split). Helium used as carrier gas was adjusted to give a column flow-rate of approximately 2 ml/min. The column was operated isothermally at 150°C for 2 min followed by a 10°C/min programme to 250°C. The temperature of the injection port, GC—MS interface and ion source were maintained at 250, 280 and 220°C, respectively. The mass spectrometer was operated in the electron impact mode with an ionization potential of 70 eV and a trap current of 200 μA. During selected ion monitoring the ions at *m/e* 361 were recorded using a Rikadenki Series DBE-6 Multi Pen recorder.

Calibration curve

For the analysis of samples with expected plasma 3-BHA levels of > 100 ng/ml the standard calibration curve was constructed with 0.1-ml blank rat plasma samples containing 5, 10, 20, 30, 40, 50 and 60 ng of 3-BHA and 40 ng 2-BHA. When the plasma 3-BHA levels were expected to be < 100 ng/ml one-tenth the above amounts of 3-BHA and internal standard were used for the calibration curve. Samples were extracted and derivatized by the method described above and subjected to GC—MS analysis. The peak height ratio of 3-BHA to 2-BHA was plotted against concentration of 3-BHA present.

RESULTS AND DISCUSSION

The heptafluorobutyryl derivatives were used in the assay procedure both to improve the GC properties of 3-BHA and to produce a suitable high mass ion for single ion detection. The electron impact mass spectrum of 3-BHA heptafluorobutyrate (Fig. 1a) exhibits both an intense molecular ion (*m/e* 376, 100%) and fragment ion at *m/e* 361 [$(M—CH_3)^+$, 93%]. The 2-BHA isomer which was selected as an internal standard for the assay showed a similar fragmentation pattern when derivatized with heptafluorobutyric anhydride (Fig. 1b). Quantitative analysis was based on recording in the electron impact mode, the ions at *m/e* 361 common to both derivatized isomers.

The GC separations were carried out on a fused silica capillary column coated with the non-polar silicone gum phase SE-52. Recent studies [10, 11] have shown that due to the very low metal oxide content of fused silica (less than 1 ppm) capillary columns fabricated from this type of glass are chromatographically very inert and exhibit good thermal stability. The flexibility and strength of the fused silica capillaries are advantages when handling and installing the columns in the GC oven. This flexibility will also allow the capillary to be inserted very close to the ion source of the mass spectrometer eliminating possible absorption or catalytic decomposition effects occurring in the GC—MS interface.

A typical GC—MS analysis with single ion detection of 3-BHA in a 0.1-ml plasma sample from a rat following an oral dosage of the antioxidant is illustrated in Fig. 2. The analyses of blank rat plasma samples gave very few background peaks at *m/e* 361 none of which interfered with the measurement of the peaks derived from 3-BHA or the internal standard. The level of detection with single ion monitoring was such that 100 pg of either of the derivatized compounds gave a signal-to-noise ratio of 10 : 1. Standard curves used for quantitation exhibited good linearity over the concentration ranges measured.

The accuracy of the method was determined from the analysis of 0.1-ml aliquots of blank plasma containing 10—100 ng of added authentic 3-BHA. The calculated recoveries over this range varied from 93.4—107.4% with a mean of 100.15% (S.D. ± 4.02). The absolute recovery of 3-BHA from plasma using 2-BHA as an external standard gave a mean recovery value of 80.3%. The precision of the assay was determined by performing replicate analyses at the concentration levels of 20, 100, 300 and 600 ng/ml plasma. The find-

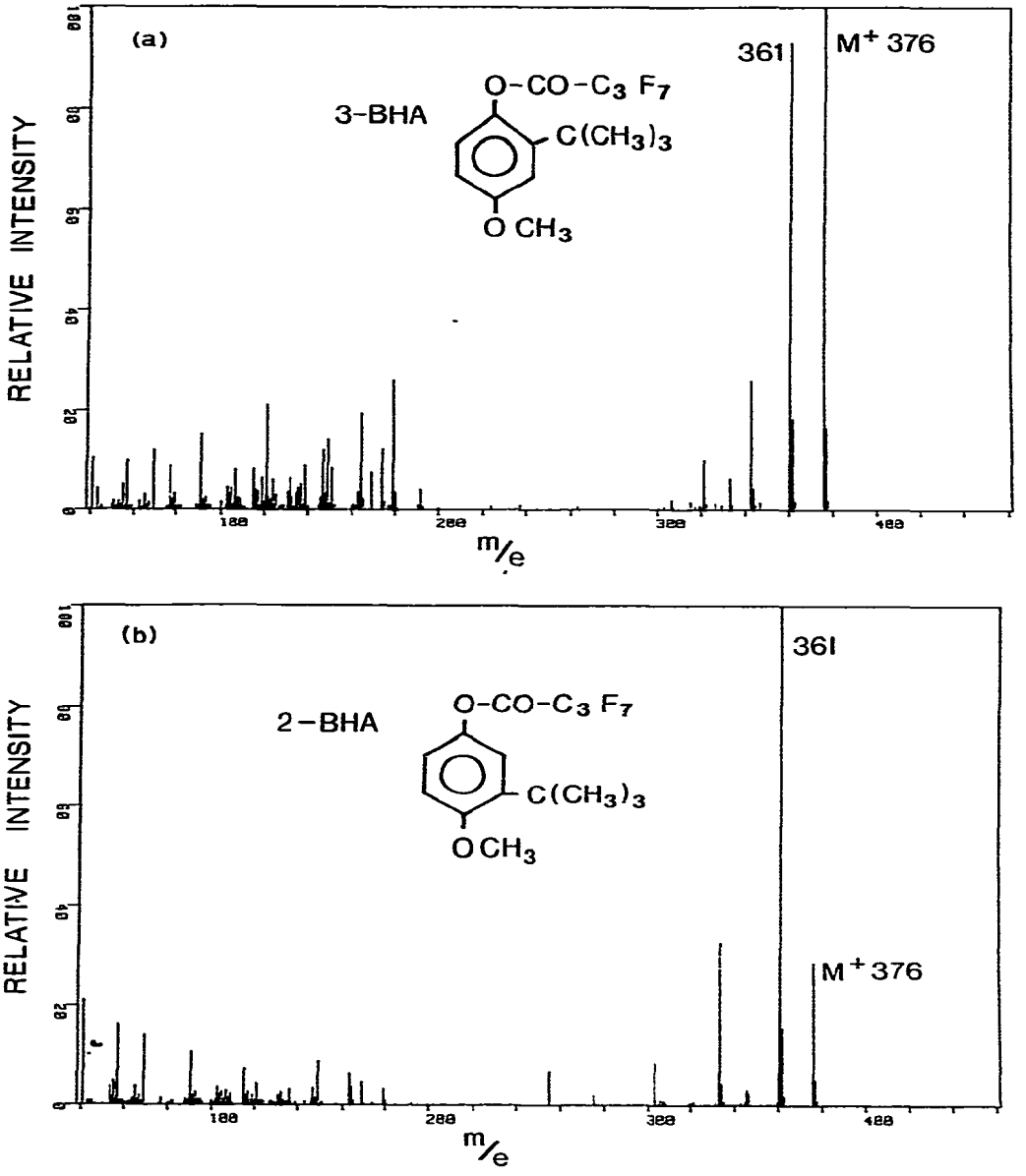
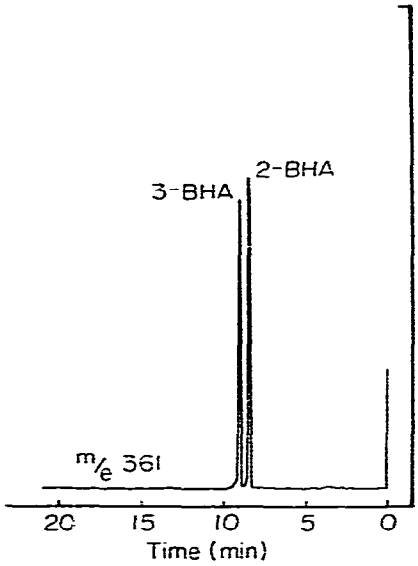


Fig. 1. Electron impact mass spectra of the heptafluorobutyryl derivative of 3-BHA (a) and 2-BHA (b).

ings are given in Table I. The specificity of the method was checked on a number of rat plasma samples by multiple ion monitoring, recording in addition to the *m/e* 361 fraction ion, the molecular ion at *m/e* 376. The peak height ratios of the two mass ions were similar to those recorded from the analysis of the standard derivatized compounds. The application of the method to a preliminary study of the plasma concentration profiles in three rats following a 200 mg/kg dose of the antioxidant is shown in Fig. 3. Following a rapid



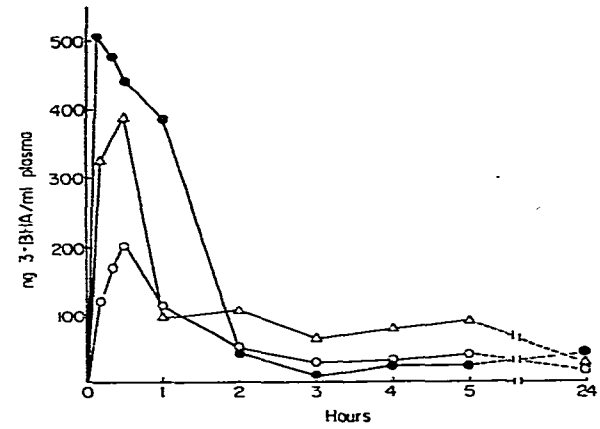


Fig. 2. Single ion monitor trace *m/e* 361 from the analysis of a plasma sample taken from a rat 2 h after a dosage of 200 mg/kg 3-BHA. The calculated concentration of 3-BHA in this sample was 65.4 ng/ml.

Fig. 3. Plasma concentration—time profiles in three rats following oral administration of 200 mg/kg of the antioxidant.

TABLE I

PRECISION OF THE METHOD FROM THE REPLICATE ANALYSES OF BLANK PLASMA SAMPLES WITH KNOWN AMOUNTS OF 3-BHA ADDED

$n = 5$.

Concentration added (ng/ml)	Mean concentration found (ng/ml)	S.D.	C.V. (%)
20	19.1	1.82	9.53
100	100.84	4.17	4.13
300	297.18	9.24	3.11
600	600.58	32.48	5.41

rise in plasma concentration, peak levels were reached at between 10 and 30 min after administration of the compound. After 2 h the levels fell below 10 ng/ml but measurable amounts were still present in the plasma 24 h after dosage.

In conclusion the method described in this paper allows the precise and accurate determination of 3-BHA at low levels in small plasma samples. The method should also be applicable to quantitating the antioxidant in urine and tissue samples.

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