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Measurement of synthetic phenolic antioxidants in human tissues by high-performance liquid chromatography with coulometric electrochemical detection

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Abstract

The antioxidants, 2-*tert.*-butyl-4-methoxyphenol (BHA) and its oxidative peroxidation product 2,2′-dihydroxy-3,3′-di-*tert.*-butyl-5,5′-dimethoxybiphenyl (di-BHA), 3,5-di-*tert.*-butyl-4-hydroxytoluene (BHT) and propyl gallate, were measured in plasma and tissue homogenates by HPLC and electrochemical detection, with a sensitivity down to 0.2 (BHA), 0.1 (di-BHA), 0.4 (BHT) and 1 (propyl gallate) ng ml^{-1} of plasma or tissue homogenate. The data demonstrate that in man, at the current level of exposure to dietary antioxidants, significant amounts of BHA, BHT and propyl gallate are accumulated in the omentum. Furthermore, they provide the first evidence that the peroxidase-catalysed oxidation of BHA is operative in man. © 1997 Elsevier Science B.V.

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1. Introduction

Butylated hydroxyanisole (2-*tert.*-butyl-4-methoxyphenol, BHA), butylated hydroxytoluene (3,5-di-*tert.*-butyl-4-hydroxytoluene, BHT) and propyl gallate are synthetic phenolic antioxidants widely used as stabilisers for fat, oils and lipid-containing foods [1].

According to the United States' Food and Drug Administration, these compounds are 'generally recognised as safe (GRAS)' as a result of their relatively low toxicity when administered orally in mammals [2,3]. In recent years, however, considerable concern has arisen, about their use as food additives, from experiments showing that BHA and BHT are potent enhancers of chemically-induced mutagenesis and carcinogenesis in laboratory animals [2–7]. Furthermore, evidence has been provided that in rodents BHA is carcinogenic in the forestomach [5,8–11] and BHT in the liver [12–14].

The FAO/WHO Joint Expert Committee on Food Additives established 'acceptable daily intakes' (ADIs) of 0–0.5 and 0–0.125 mg kg^{-1} body weight for BHA [15] and BHT [7], respectively. The

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Scientific Committee for Food of the European Communities also set an ADI of 0–0.5 mg kg^{-1} for BHA, for BHT, however, the ADI was set at the lower level of 0–0.05 mg kg^{-1} [16,17]. These ADI values were set up on a temporary basis, awaiting additional studies on the toxicity and metabolism of these compounds in various animal species to resolve the existing uncertainties [15].

An evaluation of the toxicological risk of human exposure to these additives requires information, not yet available, on the disposition of these compounds under conditions of 'normal' levels of exposure.

In recent years, a number of methods for detecting synthetic phenolic antioxidants in mammalian tissues have been developed. Methods employing radioactivity [18], gas chromatography [19,20], gas chromatography–mass spectrometry [21–24] or high-performance liquid chromatography (HPLC) with ultraviolet [25], or fluorescence detection [26,27]. Although these methods have been successfully employed for the determination of synthetic phenolic antioxidants in foods or in pharmacokinetic studies of synthetic phenolic antioxidants in experimental animals and human volunteers, they were not sensitive enough for their detection in human tissues, at the 'normal' level of exposure.

This paper describes an HPLC method with electrochemical detection for the measurement of BHA and its peroxidation product, 2,2′-dihydroxy-3,3′-di-*tert.*-butyl-5,5′-dimethoxybiphenyl (di-BHA), BHT and propyl gallate in mammalian tissues down to picogram levels.

2. Experimental

2.1. Chemicals and materials

All reagents used were of analytical grade. Chemicals were from Merck (Darmstadt, Germany) except as follows: BHT and diethyl ether were obtained from Fluka (Buchs, Switzerland); propyl gallate and DBHA (3,5-di-*tert.*-butyl-4-hydroxyanisole) were purchased from Aldrich (Brussels, Belgium); 4-butoxyphenol, 4-pentyloxyphenol and 4-hexylresorcinol were purchased from Ega-Chemie (Steinheim/Albhruch, Germany); BHA and BHT were recrystallized once from petroleum ethanol and ether, respectively; the dimer of BHA, di-BHA was synthesised by direct oxidation of BHA as previously described [28]; diethyl ether was stabilised with 2,6-di-*tert.*-butyl-*p*-cresol which did not interfere with the separation of the studied compounds.

2.2. Apparatus

Isocratic HPLC determination was carried out with a Shimadzu LC-6A single piston pump (A.STR.AN, Milan, Italy), an ESA 5100A coulochem detector with an ESA 5011 analytical cell containing dual working electrodes (Italscientifica, Genova, Italy), and a Shimadzu Chromatopac C-R4A Integrator (A.STR.AN, Milan, Italy). Separation by isocratic elution was carried out on reversed-phase Nucleosil C_8 or C_{18} (200×4 mm) column, particle size 5 μm (Macherey-Nagel, Durem, Germany). Samples were injected with a Rheodyne 7125 (Cotati, CA, USA) syringe loading injector (20-μl loop).

2.3. Mobile phase

The mobile phase was methanol–water at different percentages, depending on the compound analysed and on the column used for separation. The mobile phase was adjusted to pH 3.4 with 85% H_3PO_4 or 100% glacial acetic acid, filtered through a 22-μm filter (Millipore, Bedford, MA, USA) and degassed for 2 min by ultrasound before use. A flow-rate of 0.7 ml min^{-1} at ambient temperature was used.

2.4. Human studies

Tissue fragments of omentum operative specimens were obtained from 50 patients (20 females, mean age 69±4 years and body weight 60±3 kg; 30 males, mean age 59±3 years and body weight 73±3 kg) undergoing various surgical gut treatments. Tissue homogenates were prepared as previously described [24]. Tissue fragments were washed (0.9% NaCl), blotted, weighed, minced using an Ultraturrax apparatus, and then homogenised into 3 volumes of 0.1 *M* sodium phosphate buffer (pH 7.2). All 50 omentum homogenates were processed for the analysis of BHA, 49 for that of di-BHA, whereas the analysis of BHT and propyl gallate was performed only in 42 of the 50 omentum samples. Human

plasma samples (45) were obtained from the hospital blood bank, all of them where analysed for each of the four compounds.

For the analysis of human plasma and omentum homogenates, calibration curves were constructed by spiking 'blank' tissue samples, taken from rats maintained on an antioxidant-free diet, with increasing amounts of the phenolic compounds, together with a constant amount of their corresponding internal standard, when available.

2.5. Preparation of samples

BHA and di-BHA were extracted from plasma and omentum homogenates by a modification of the method previously described [24]. Samples of plasma or homogenate (500 μl) were mixed with 60 μl of methanol containing 4-butoxyphenol or DBHA. As discussed later, 4-butoxyphenol was found to be a satisfactory internal standard for BHA, as DBHA was for both di-BHA and BHT. Samples were then extracted with diethyl ether (2.5 ml) by mechanical shaking (10 min) and centrifuged (5 min at 1000 *g*). After removal of the aqueous phase by aspiration, the organic layer was evaporated to dryness, at room temperature under a nitrogen stream. The residue was then dissolved in methanol (60 μl) and vortexed prior to injection (20 μl). Our earlier work using GLC–mass spectrometric (MS) analysis had shown that plasma and tissue homogenates could be stored for more than a year at −25°C without loss of BHA and di-BHA [29].

BHT and propyl gallate were extracted from plasma and omentum homogenates (500 μl), obtained as described above, after mixing with 60 μl of methanol containing DBHA as the internal standard for BHT, whereas no adequate internal standard was found for propyl gallate (see below). Both compounds were extracted with 2.5 ml of diethyl ether but, in the case of BHT, the diethyl ether was enriched with corn oil (1.4%) to reduce the higher volatility of this compound [26,27]. After mechanical shaking (10 min) and centrifugation (5 min at 1000 *g*), for both BHT and propyl gallate extraction, samples underwent an additional washing step with 500 μl of water and were then centrifuged again. The organic layer was then removed, transferred into pointed centrifuge tubes and evaporated to dryness at 0°C, under a nitrogen stream. The residue was dissolved in 60 μl methanol, vortexed and centrifuged for 5 min before injection (20 μl). In the present study we found that plasma and tissue homogenates could be stored for a year at −25°C without loss of BHT and propyl gallate.

3. Results and discussion

A good separation of BHA, di-BHA, BHT and propyl gallate was obtained with either C_8 or C_{18} stationary phases. The C_{18} phase was preferred as it was found to have a greater long-term stability under the conditions used here. The methanol–water (v/v) mixtures, used as isocratic mobile phases with the C_8 and C_{18} columns, and the corresponding retention times (t_R) of BHA, di-BHA, BHT, propyl gallate and their internal standards are shown in Table 1. Among

Table 1
Methanol–water (v/v) mixtures used as isocratic mobile phases with C_8 and C_{18} columns, and corresponding retention times of BHA, di-BHA, BHT, propyl gallate and their internal standards, 4-butoxyphenol and DBHA

Compound (internal standard)	C_8 Methanol–water (v/v)	C_8 t_R (min)	C_{18} Methanol–water (v/v)	C_{18} t_R (min)
BHA	59:41	8.0	69:31	9.7
(4-Butoxyphenol)		6.5		7.5
di-BHA	76:24	7.0	83:17	6.5
(DBHA)		5.0		5.5
BHT			76:24	14.5
(DBHA)				9.0
Propyl gallate			46:54	7.5

t_R=retention time; BHA=2-*tert.*-butyl-4-methoxyphenol; di-BHA=2,2′-dihydroxy-3,3′-di-*tert.*-butyl-5,5′-dimethoxybiphenyl; BHT=3,5-di-*tert.*-butyl-4-hydroxytoluene; DBHA=3,5-di-*tert.*-butyl-4-hydroxyanisole.

several derivatives tested (4-butoxyphenol, 4-pentyloxyphenol and 4-hexylresorcinol), 4-butoxyphenol was chosen as a suitable internal standard for BHA. This was because (1) it gave a good separation at a shorter retention time and was closer to the elution time of BHA, and (2) the detector response was identical to that of BHA (see Fig. 1A). DBHA was a good internal standard for both di-BHA and BHT, whereas in the case of propyl gallate a suitable internal standard could not be found since the low methanol percentage in the mobile phase resulted in the compounds tested as possible candidates (dodecyl gallate, octyl gallate and *p*-butoxyphenol) being eluted after 30 min. Although it would be possible to develop a system involving gradient elution with a C_{18} column to resolve all these antioxidants with their metabolites, a more robust system was obtained by the use of a C_{18} column and isocratic elution under different conditions for each of the antioxidants. Representative chromatograms obtained running human omentum samples, containing detectable amounts of BHA, di-BHA, BHT and propyl gallate, on a C_{18} column are shown in Fig. 2, panels A, B, C and D, respectively.

The electrochemical detector system was characterised by a coulometric guard cell, used for pre-

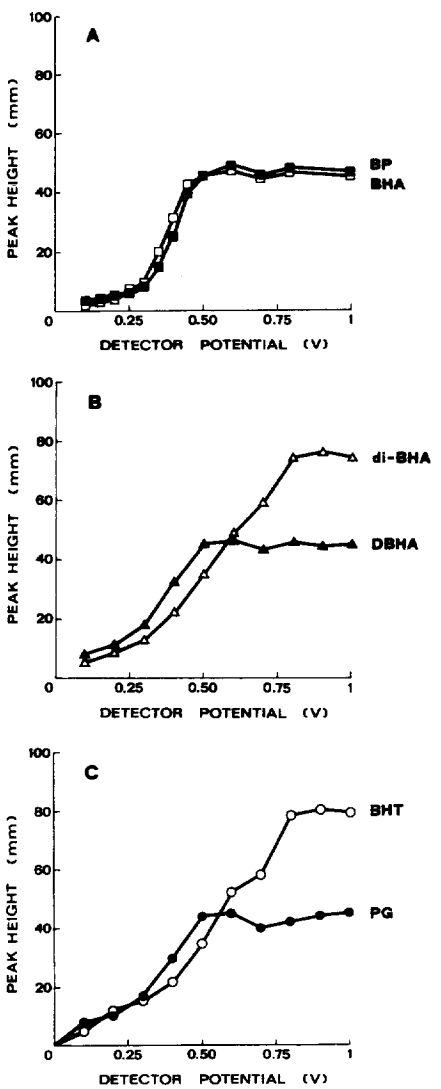

Fig. 1. Voltammograms of BHA (2-*tert.*-butyl-4-methoxyphenol) and 4-butoxyphenol (panel A), di-BHA (2,2′-dihydroxy-3,3′-di-*tert.*-butyl-5,5′-dimethoxybiphenyl) and DBHA (3,5-di-*tert.*-butyl-4-hydroxyanisole) (panel B), BHT (3,5-di-*tert.*-butyl-4-hydroxytoluene) and propyl gallate (panel C).

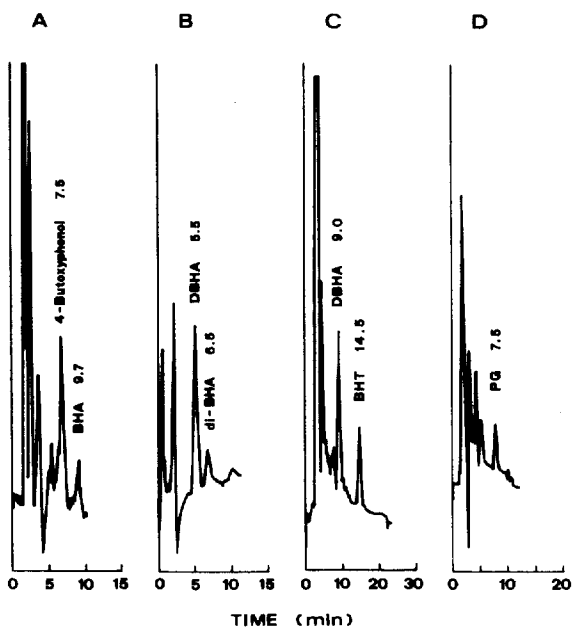

Fig. 2. Typical chromatograms obtained from processed human omentum homogenate samples. A sample spiked with 4 ng ml^{-1} of the internal standard 4-butoxyphenol, where BHA was estimated to be 1.64 ng ml^{-1}, is shown in Panel A; samples spiked with 11.6 ng ml^{-1} of the internal standard DBHA, where di-BHA was estimated to be 0.73 ng ml^{-1} (panel B) and BHT 1.78 ng ml^{-1} (panel C); and finally a sample were propyl gallate was estimated to be 2.43 ng ml^{-1} is shown in Panel D.

Table 2
Concentration range, mean of slopes and their average standard deviation (S.D.) for calibration curves (n=3–7) obtained on different days from blank plasma samples spiked with 4–6 concentrations of BHA, di-BHA, BHT and propyl gallate

Compound	Concentration range (ng ml^{-1})	Slope (10^3)		r
		Mean	Average S.D.	
BHA	0.2–80	0.877	0.063	>0.99
di-BHA	0.1–50	0.922	0.081	>0.99
BHT	0.4–80	0.409	0.047	>0.98
Propyl gallate[a]	1.0–80	17.620	1.700	>0.99

BHA=2-*tert.*-butyl-4-methoxyphenol; di-BHA=2,2′-dihydroxy-3,3′-di-*tert.*-butyl-5,5′-dimethoxybiphenyl; BHT=3,5-di-*tert.*-butyl-4-hydroxytoluene.
Results obtained with blank omentum and intestine homogenates were not significantly different.
[a] Without internal standard.

oxidation of mobile phase as a preventive measure against the possible presence of contaminants, and dual coulometric working electrodes. The first electrode was set at 0.02–0.08 V to oxidise undesirable endogenous electroactive compounds, whereas the oxidation potential of the second electrode required for each compound was determined from their voltammograms. The hydrodynamic voltammograms for BHA (Fig. 1A), di-BHA (Fig. 1B), BHT and propyl gallate (Fig. 1C) and their respective internal standards indicated that the electrode potential for maximal detector response of BHA and 4-butoxyphenol was 0.6 V, whereas the oxidation potential required for maximal di-BHA and BHT response was 0.8 V, which was higher than that for maximal response of their internal standard DBHA (0.6 V). The maximal electrode response of propyl gallate was obtained at an oxidation potential of 0.5 V.

The calibration curves exhibited a good linearity for all compounds examined, over the concentration range used, showing intersections with the y-axis not significantly different from the origin. Furthermore, it was found that the linearity of calibration curves was unaffected by omission of the internal standard. Ranges and average standard deviations of slopes for standard curves obtained on different days, from four to six known concentrations each day, are shown in Table 2. The average between-assay coefficients of variation (C.V.) were below 10% and the difference between the found and nominal concentration ranged from −3.7% to 6.3%, for the minimum detectable concentration of BHA, di-BHA, BHT and propyl gallate shown in Table 3. Recoveries of the compounds (Table 3) added to plasma and tissue homogenates were calculated by comparing peak areas with those obtained injecting the corresponding

Table 3
Accuracy, precision and recovery of the method using 3–9 replicate analyses of blank plasma samples spiked with the minimum detectable concentration of BHA, di-BHA, BHT and propyl gallate

Compound	Precision			Accuracy (C.V. %)	Recovery (%)
	Concentration (ng ml^{-1})		Difference (%)		
	Nominal	Found			
BHA	0.270	0.260	−3.7	8.2	80.2±8.0
di-BHA	0.135	0.140	+3.8	9.8	87.3±6.3
BHT	0.525	0.555	+5.7	8.9	76.9±5.9
Propyl gallate[a]	1.060	1.127	+6.3	9.5	44.8±8.0

BHA=2-*tert.*-butyl-4-methoxyphenol; di-BHA=2,2′-dihydroxy-3,3′-di-*tert.*-butyl-5,5′-dimethoxybiphenyl; BHT=3,5-di-*tert.*-butyl-4-hydroxytoluene.
Results obtained with blank momentum and intestine homogenates were not significantly different.
[a] Without internal standard.

amount directly added to methanol. Mean recoveries of BHA, di-BHA and BHT ranged from 77 to 87% in all cases except for propyl gallate where a lower recovery (45%) was obtained. No statistically significant difference (ANOVA, $P<0.05$) in recoveries were observed between plasma and homogenates of different tissues, omentum and intestine.

The method was applied to the determination of the three additives, BHA, BHT and propyl gallate in samples of human plasma and omentum. BHA was detected in 30 out of the 45 plasma samples analysed and these 30 showed a mean value of 1.02 ± 0.17 ng ml^{-1} (Fig. 3), whereas neither BHT nor propyl gallate could be detected in any of them. All three antioxidants, however, were detected in at least some of the omentum samples analysed. Detectable omentum levels of BHA, BHT and propyl gallate were found in 66% (33 out of 50), 64% (27 out of 42) and 30% (13 out of 42), respectively, of the analysed patients.

Despite of the wide use of these antioxidants as food additives, information about their intake is extremely limited and indirect. Verhagen et al. [30] have conducted a dietary record survey providing

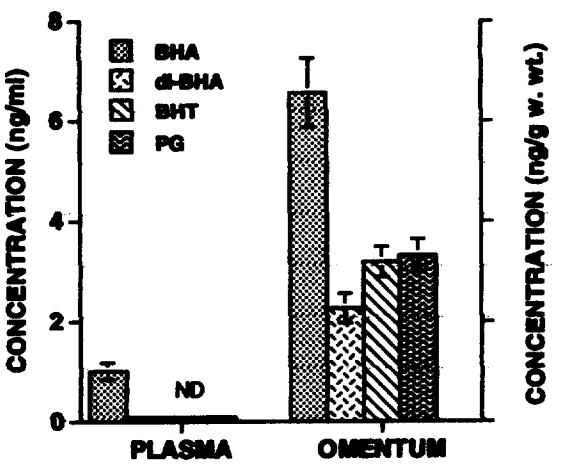

Fig. 3. Levels of BHA (2-*tert.*-butyl-4-methoxyphenol), di-BHA (2,2′-dihydroxy-3,3′-di-*tert.*-butyl-5,5′-dimethoxybiphenyl), BHT (3,5-di-*tert.*-butyl-4-hydroxytoluene) and propyl gallate detected in plasma and omentum homogenates. Columns represent mean values (bars=±S.E.M.) of those samples where levels were detectable. In plasma BHA was detected in 30 out of 45 samples analysed; in omentum BHA, di-BHA, BHT and propyl gallate were detected in 33/50, 25/49, 27/42 and 13/42, respectively. ND=non detectable.

estimates of the maximal dietary intake of BHA and BHT in the Netherlands in 1987/88. Their estimates were based on the fat content of selected food categories and their respective maximum permitted levels of these antioxidants. The present HPLC method is sufficiently sensitive, accurate and precise to provide direct information on the presence of BHA, BHT and propyl gallate in human tissue specimens.

Since earlier studies performed in this laboratory have shown that in the rat BHA is transformed in vivo into its peroxidation product di-BHA [24], which may then be retained in the tissues for long periods, it was of interest to verify the possible presence of this metabolite in human tissues. Indeed the peroxidative metabolite of BHA, di-BHA, although undetectable in plasma, was found to be present in the omentum of 51% of the patients analysed (25 out of 49).

In conclusion the present data demonstrate that in man, at the current level of exposure to dietary antioxidants, significant amounts of BHA, BHT and propyl gallate are accumulated in the omentum. Furthermore, they provide the first evidence that the peroxidase-catalysed oxidation of BHA is operative in man.

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