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Note

Determination of butylated hydroxytoluene in plasma by high-performance liquid chromatography

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Butylated hydroxytoluene (3,5-di-*tert.*-butyl-4-hydroxytoluene, BHT, Fig. 1) and butylated hydroxyanisole (2-(3-)*tert.*-butyl-4-hydroxyanisole, BHA, Fig. 1) are phenolic anti-oxidants widely used in foods and technical products [1–3]. The toxicity of BHT in animals is well documented [2–4]. It has been shown that rodent liver and lungs are especially susceptible to the toxic effects of BHT [5,6]. BHT interferes with blood coagulation and causes haemorrhagic death in rats [7]. Furthermore, BHT and BHA appear to play a dual role in the modification of chemical carcinogenesis, exhibiting pro- as well as anti-carcinogenic properties [8,9]. The incidence of mild hyperplasia in hamster forestomach was slightly higher after oral BHT intake [10]. BHT acts as a full carcinogen in lungs and liver of rodents [9,11,12].

Analysis of the in vivo kinetics and metabolism of BHT may contribute to the evaluation of its potential health risk. In this respect, only a few methods for the determination of BHT in plasma or serum have been reported, applying radioactivity [13], gas chromatography (GC) [14,15], gas chromatography–mass

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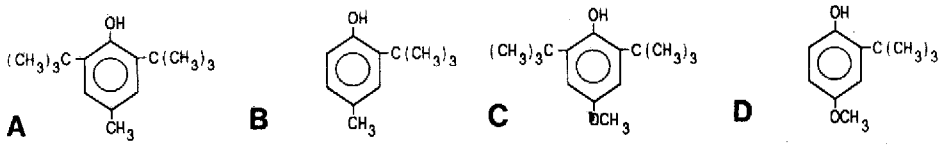

Fig. 1. Structures of (A) 3,5-di-*tert.*-butyl-4-hydroxytoluene (BHT), (B) 3-mono-*tert.*-butyl-4-hydroxytoluene (MBHT), (C) 3,5-di-*tert.*-butyl-4-hydroxyanisole (DBHA) and (D) 3-*tert.*-butyl-4-hydroxyanisole (3-BHA).

spectrometry (GC–MS) [16] or high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection and gradient elution [17].

We recently reported a sensitive method for the routine determination of butylated hydroxyanisole in plasma [18]. This paper describes a simple procedure for the routine determination of BHT in plasma by means of reversed-phase HPLC with isocratic elution and fluorescence detection.

EXPERIMENTAL

Chemicals and materials

BHT and BHA were obtained from Sigma (St. Louis, MO, USA); DBHA (3,5-di-*tert.*-butyl-4-hydroxyanisole, Fig. 1) and MBHT (3-mono-*tert.*-butyl-4-hydroxytoluene, Fig. 1) were purchased from Aldrich (Brussels, Belgium). Acetonitrile (HPLC grade) was purchased from Rathburn (Walkerburn, U.K.). Diethyl ether, stabilized with pyrogallol instead of BHT, was obtained from BDH (Poole, U.K.). Water was purified using a Milli-Q water purification system. Analytical-reagent grade chemicals were used in all other instances. Plasma prepared from fresh citrated bovine blood was used for constructing calibration graphs.

Preparation of samples

Aliquots of plasma (0.2–1.0 ml, spiked with 0–5000 ng/ml BHT for constructing calibration graphs) were diluted to 1.0 ml with water. A 25-μl aliquot of a solution of 100 μg/ml DBHA in methanol was added as an internal standard. Samples were subsequently extracted in a glass-stoppered tube with 5 ml of oil-enriched diethyl ether (one drop of corn oil in 200 ml diethyl ether). The samples were shaken for 0.5 h and centrifuged at 700 *g* for 5 min. The organic phase was separated and evaporated to dryness at 0°C under a gentle stream of nitrogen. The residue was dissolved in 0.5 ml methanol, 0.5 ml of concentrated hydrochloric acid was added, and the stoppered tubes were incubated for 1 h at 95°C in a shaking water-bath in order to de-alkylate BHT and DBHA to their strongly fluorescent counterparts MBHT and 3-BHA (Fig. 1), respectively. After cooling on ice, the samples were diluted with 2.5 ml water, extracted for 0.5 h with 5 ml diethyl ether, centrifuged at 700 *g* for 5 min and evaporated to dryness at 0°C under a gentle stream of nitrogen. The residue was dissolved in 500 μl of eluent and stored prior to analysis.

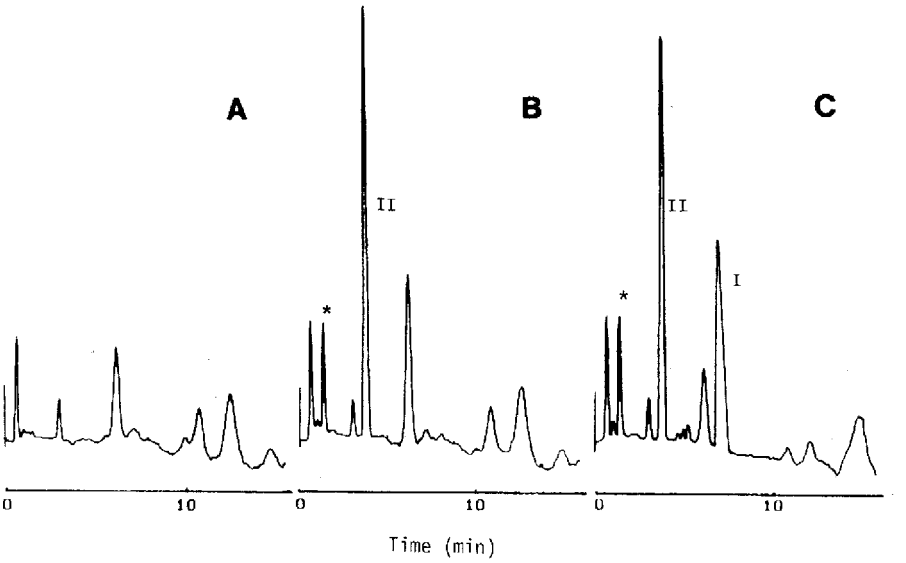

Fig. 2. Chromatograms for the assay of BHT in plasma using DBHA as an internal standard. The de-alkylated products MBHT (I) and BHA (II) are indicated. (A) Extract of 1 ml blank plasma; (B) extract of 1 ml blank plasma with internal standard; asterisk indicates an unidentified product formed out of DBHA during the assay procedure; (C) extract of 200 μl of rat plasma containing 3.1 μg/ml BHT. Chromatograms were recorded using a programmable fluorescence detector set at 0.050 A.U. between 0 and 5 min and at 0.010 A.U. between 5 and 15 min. Auto-zeros were given at $t=0$ and $t=5$ min.

High-performance liquid chromatography

HPLC analysis was performed with a Kratos Spectroflow 400 pump and a Kratos Spectroflow 980 programmable fluorescence detector. A LiChrosorb RP-18 cartridge column and appropriate guard column (Chrompack, Middelburg, The Netherlands) were used. The excitation wavelength was set at 273 nm and a cut-off filter at 320 nm was used. Samples of 100 μl were injected with a Rheodyne 7125 sample injector. The solvent, acetonitrile–water–acetic acid (45:54:1, v/v/v), was degassed prior to use in an ultrasonic bath and recycled at a flow-rate of 1.0 ml/min.

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF BHT IN PLASMA SAMPLES SPIKED AT THREE DIFFERENT CONCENTRATIONS

Added concentration (ng/ml)	*n*	Measured concentration (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Accuracy (%)	Recovery (%)
50	3	51 ± 4	7	102 ± 7	100 ± 10
500	3	460 ± 10	2	92 ± 2	75 ± 1
5000	3	5106 ± 187	4	102 ± 4	70 ± 2

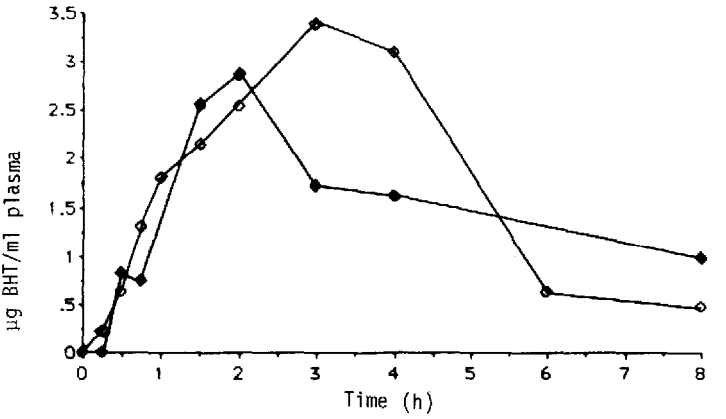

Fig. 3. BHT concentrations in plasma of two rats sampled at various times after in gastro administration of 200 mg/kg BHT in corn oil.

Animal experiments

A catheter was placed under ether anaesthesia into the right femoral artery of two male Wistar rats (294 and 300 g; Winkelmann, Borchem, F.R.G.). After fasting overnight the rats received an oral dose of 200 mg/kg BHT, as a solution of 200 mg/ml BHT in corn oil. Blood was sampled at 0, 15, 30, 45, 60, 90, 120, 180, 240, 360 and 480 min after administration. Blood samples of 400–450 μl were collected in heparanized vessels and centrifuged at 2000 *g* for 5 min. Prior to analysis, plasma was stored at −20°C. Washed blood cells (0.5 ml) mixed with 0.5 ml saline were readministered after 90 and 180 min in order to protect the animals from anaemia.

RESULTS AND DISCUSSION

Extraction and chromatography

A few methods for the determination of BHT in plasma or serum have been reported. However, only one HPLC procedure is available [17], which uses gradient elution and UV detection, with a limit of detection of about 100 ng/ml serum. We have tried to develop a more sensitive HPLC method, using an isocratic elution system. As already reported for BHA [18], phenolic anti-oxidants, due to their volatility, may be lost during sample preparation. In our experience, BHT and especially MBHT are much more volatile than BHA and DBHA. Therefore it was not only necessary to perform the evaporation of the extraction solvent at 0°C, but also to add a little oil. BHT itself has no fluorescent properties [19], unlike the dealkylated product MBHT. Therefore, we modified the procedure of Mizutani and Ohe [20]. BHT and DBHA were de-alkylated to their monobutyl counterparts MBHT and 3-BHA, respectively, using concentrated hydrochloric acid.

The retention times of BHA and MBHT were 3.6 and 7.0 min, respectively. The total run time was 15 min. Typical chromatograms are shown in Fig. 2. Blank plasma extracts (Fig. 2A) only revealed a small peak at the MBHT location,

independent of the amount of plasma analyzed. This probably originated from some unknown impurity introduced during the procedure. Therefore, the practical limit of detection is about 20 ng BHT per ml plasma (1-ml samples) or 4 ng absolute per injection. Calibration graphs, calculated from peak-height ratios, were linear over the range 0–500 ng/ml BHT ($y=0.00152x+0.030$, $r=0.9952$, $n=3$) and 0–5000 ng/ml BHT ($y=0.00130x+0.060$, $r=0.9964$, $n=3$). The absolute recovery of BHT on a molar basis after extractions and transformation into MBHT is shown in Table I. The absolute recovery of DBHA as BHA on a molar basis was $56\pm3\%$ (mean $\pm$S.D.). Upon treatment with concentrated hydrochloric acid in methanol, BHA and at least one unidentified product, indicated by the asterisk in Fig. 2, were formed out of DBHA. This may be an explanation for the relatively low, but reproducible recovery of DBHA in our procedure. However, the recovery of the internal standard proved to be constant over a broad concentration range (results not shown). The variability of the assay is shown in Table I. These results are better than, or comparible with other reported methods involving radioactivity, GC, GC–MS or HPLC [12–16].

Animal experiments

Concentration versus time curves for BHT in the plasma of two rats after oral administration are presented in Fig. 3. A peak concentration of about 3 μg/ml was observed after 2–3 h. These preliminary data are not in agreement with the results of Rao and Wong [15], who found plasma concentrations peaking between 250 and 450 μg/ml in a similar experiment. In an experiment with BHA instead of BHT, we showed that peak concentrations of BHA in plasma were between 0.2 and 1.0 μg/ml [18], which indicates that BHT is more rapidly absorbed from the gastro-intestinal tract and/or more slowly cleared from plasma, as compared to BHA. Detailed toxicokinetic studies of BHT in rats are currently being undertaken.

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