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Note

Sensitive high-performance liquid chromatographic method for the routine determination of butylated hydroxyanisole in plasma

HANS VERHAGEN

Department of Human Biology, State University of Maastricht, Biomedical Centre, P.O. Box 616, 6200 MD Maastricht (The Netherlands)

HENK H.W. THIJSSEN

Department of Pharmacology, State University of Maastricht, Biomedical Centre, P.O. Box 616, 6200 MD Maastricht (The Netherlands)

and

JOS C.S. KLEINJANS*

Department of Human Biology, State University of Maastricht, Biomedical Centre, P.O. Box 616, 6200 MD Maastricht (The Netherlands)

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Butylated hydroxyanisole [2-(3-)*tert.*-butyl-4-hydroxyanisole, BHA, Fig. 1] is widely used in foods, cosmetics and plastics for its radical-trapping and therefore antioxidative properties [1–3]. Owing to the large-scale use of BHA, its possible toxicity to man is of great interest. Earlier studies regarded the application of BHA as a food additive to be safe, especially when considering its expected low dietary intake [4]. Further, BHA was observed to act as an anticarcinogen in various animal carcinogenesis models [2,5]. However, chronic feeding of BHA at relatively high doses has recently been reported to induce hyperplasia in rodent forestomach [6] and to increase mitotic index in primate oesophagus [7], thus revealing some carcinogenic potential.

Further evaluation of the toxicological properties of BHA demands a critical assessment of its toxicokinetic characteristics. However, only a few methods for the determination of BHA in animal and human plasma are available, all of them involving gas chromatography (GC) [8,9] or gas chromatography–mass spec-

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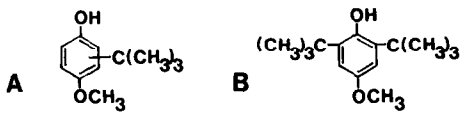

Fig. 1. Structures of (A) 2-(3-)*tert.*-butyl-4-hydroxyanisole (BHA) and (B) 2,5-di-*tert.*-butyl-4-hydroxyanisole (DBHA).

trometry (GC–MS) [10–12]. There is no uniformity in the extraction procedure for BHA from plasma. Further, a routine high-performance liquid chromatographic (HPLC) method for the determination of BHA in plasma has not yet been established.

This paper describes a simple and rapid procedure for the extraction of BHA from plasma and its quantitation by means of reversed-phase HPLC with fluorescence detection.

EXPERIMENTAL

Chemicals and materials

BHA (3.5–4% 2-*tert.*-butyl-4-hydroxyanisole plus 94–96% 3-*tert.*-butyl-4-hydroxyanisole) was obtained from Sigma (St. Louis, MO, U.S.A.) and DBHA (2,5-di-*tert.*-butyl-4-hydroxyanisole) (Fig. 1) from Aldrich (Brussels, Belgium). Methanol (HPLC grade) and diethyl ether were purchased from Merck (Darmstadt, F.R.G.). Water was purified using a Milli-Q water purification system. Analytical-reagent grade chemicals were used in all other instances.

Pool plasma was prepared from fresh citrated bovine blood, obtained from a neighbourhood slaughterhouse.

Extraction of plasma

Pool plasma was spiked with known amounts of BHA in methanol to achieve plasma concentrations in the range 0–100 μg/ml. The final methanol concentration never exceeded 1%.

A 0.1–1.0-ml volume of this BHA-loaded plasma was diluted to 1.0 ml with water and subsequently extracted in a glass-stoppered tube with 5.0 ml of diethyl ether containing 0.4 μg/ml DBHA as an internal standard. The samples were shaken for 1 h and centrifuged at 700 *g* for 5 min. A 4.5-ml volume of the organic phase was evaporated to dryness at 0°C under a gentle stream of nitrogen. The residue was dissolved in 90 μl of methanol and retained until analysis.

High-performance liquid chromatography

The HPLC system consisted of a Kratos Spectroflow 400 pump and a Kratos Spectroflow 980 programmable fluorescence detector with an excitation wavelength of 290 nm and a cut-off filter at 320 nm. Injections were performed via a 20-μl sample loop of a Rheodyne 7125 syringe loading sample injector.

A LiChrosorb 5 RP-18 (150 mm × 4.6 mm I.D.) (Chrompack, Middelburg, The Netherlands) was used in combination with a guard column (ODS pellicular material) (30 mm × 2.1 mm I.D.). The mobile phase, modified from the method

of Archer [13], consisted of 1% acetic acid in methanol–water (73:27, v/v). The eluent, which had been degassed prior to use in an ultrasonic water-bath, was recycled at a flow-rate of 2.0 ml/min and kept at 40 °C to prevent the introduction of air.

The two isomeric forms of BHA are not separated by this procedure.

Animal experiments.

Two male Wistar rats (307 and 318 g) (TNO, Rijswijk, The Netherlands) received a catheter into the right femoral artery under ether anaesthesia and 24 h after surgery the rats, fasted overnight, were given by gavage 1 ml/kg of a solution containing 200 mg/ml BHA in dimethylsulphoxide (DMSO) [11]. Blood samples of 200–250 μl were taken from the femoral artery after 0 (control), 10, 20, 30, 50, 70, 90, 120, 180, 240 and 360 min, collected in heparinized vessels, and centrifuged for 5 min at 2000 *g*. Plasma was stored at −20 °C until taken for analysis. In order to protect the animals from anaemia, 1 ml of washed blood cells, diluted 1:1 with saline, was re-administered after the 120-min blood sample had been taken.

RESULTS AND DISCUSSION

Extraction procedure

Several procedures for the extraction of BHA from animal or human plasma have been described [8–12]. Dichloromethane, hexane and light petroleum have been applied for this purpose, and diethyl ether was used to extract BHA from urine [14]. In our experience, several solvents (dichloromethane, chloroform, hexane, ethyl acetate, diethyl ether) are suitable for the extraction of BHA from plasma.

However, because phenolic antioxidants are volatile [3], evaporation to dryness after extraction appeared to be the most delicate step in the procedure. Indeed, most of the amounts of BHA and DBHA dissolved in methanol, which was evaporated under nitrogen at 40 °C, could be trapped in a filter filled with activated charcoal and placed on top of the evaporation tube, the filter subsequently being eluted with methanol and the extract analysed by HPLC. Since, in a separate experiment, BHA and DBHA could be retained by means of a tiny drop of oil added to the extraction solvent which was evaporated at 40 °C, it is suggested that the amount of co-extracted lipid material from plasma contributes to avoiding sublimation of BHA and DBHA at higher temperatures. To avoid this volatility phenomenon of BHA, in our study plasma extracts were evaporated at 0 °C and diethyl ether was chosen as the extraction solvent because of its ease of evaporation at 0 °C.

Chromatography

The retention times of BHA and DBHA were 1.9 and 5.1 min, respectively, and the total run time was 12 min. We have found that fluorescence detection is both selective and sensitive for BHA. Typical chromatograms are shown in Fig. 2. Blank plasma extracts did not reveal any peaks at the locations of BHA or DBHA.

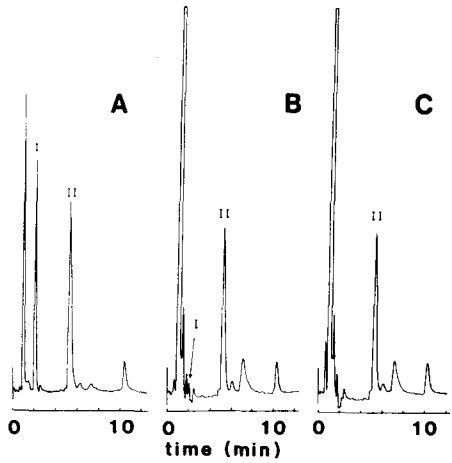

Fig. 2. Chromatograms of BHA (I) assay in plasma using DBHA (II) as an internal standard. (A) Extract of 100 μl of plasma spiked with 1000 ng/ml BHA; (B) ten-fold concentrated extract of 1 ml of plasma spiked with 10 ng/ml BHA; (C) ten-fold concentrated extract of 1 ml of blank plasma. Chromatograms were recorded using a programmable fluorescence detector with a range set at 0.007 A.U. between 0 and 2.5 min and at 0.020 A.U. between 2.5 and 12 min. Auto-zeros were given at $t=0$, 1.6, 2.5 and 4.8 min.

Calibration graphs, calculated from peak-height ratios, showed good linearity over the range 0–100 μg/ml BHA (Fig. 3). The recovery of BHA, calculated from spiked 500 and 1000 ng/ml plasma samples, was $90 \pm 6\%$ (mean $\pm$ S.D., $n=10$). The limit of detection of BHA was 1 ng absolute (signal-to-noise ratio = 5). In our plasma samples concentrations up to 10 ng/ml could be assayed accurately (Fig. 2B). The coefficient of variation for the assay procedure as determined for spiked 0.5 and 100 μg/ml samples was 4–5% ($n=8$). These results are compatible with reported methods involving GC [8,9] or GC–MS [10–12].

Animal experiments

Plasma concentration–time curves for BHA in two rats following its oral administration are presented in Fig. 4. In one rat a plasma BHA peak concentra-

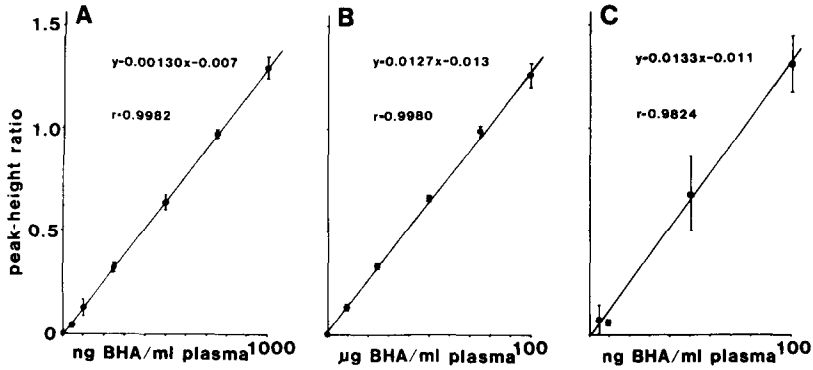

Fig. 3. Calibration graphs for BHA extracted from spiked plasma samples using DBHA as an internal standard. (A) Range 0–1000 ng/ml BHA, 100-μl samples, $n=4$; (B) range 0–100 μg/ml BHA, 100-μl samples, $n=4$; (C) range 0–100 ng/ml BHA, ten-fold concentrated 1-ml samples, $n=3$.

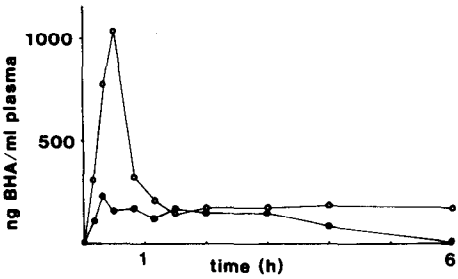

Fig. 4. BHA concentrations in plasma of two rats sampled at various times after in gastro administration of 200 mg/kg BHA in DMSO.

tion was observed at 30 min; no BHA peak was observed in the other rat. The elimination of BHA from plasma seemed to be slow. These preliminary data are in agreement with those of Bailey et al. [11]. Detailed toxicokinetic studies of BHA in the rat are currently being undertaken.

To our knowledge this is the first reported HPLC method for the determination of BHA in plasma, which in our hands proved also to be applicable to the determination of BHA in urine.

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