Journal of Chromatography, 342 (1985) 129—134
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2604

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE ANTITUMOR GLYCOSIDE PHYLLANTHOSIDE AND ITS STABILITY IN PLASMA OF SEVERAL SPECIES

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(Received December 4th, 1984)

SUMMARY

Phyllanthoside is a glycoside isolated from the roots of the Central American tree *Phyllanthus acuminatus* Vahl with antitumor activity against murine B-16 melanoma and P-388 leukemia. We report a reversed-phase high-performance liquid chromatographic assay for phyllanthoside in plasma using a 25-cm RP-18, 5-μm column with a linear 10-min gradient of 50% to 100% methanol in 0.3 *M* sodium acetate, pH 4.0, at a flow-rate of 1.5 ml/min. Eluting peaks were detected at 270 nm. The lower limit of sensitivity of the assay for phyllanthoside in 0.5 ml plasma following ethyl acetate extraction at pH 7.0 was 0.25 μg/ml and the coefficient of variation at 1 μg/ml was ± 7.4%. Phyllanthoside was very rapidly broken down by mouse and rat plasma in vitro to an unidentified less polar metabolite. Formation of this metabolite was completely inhibited by preheating mouse plasma to 100°C for 10 min. When mouse plasma was diluted 1:50 with water the half-life of phyllanthoside disappearance at 37°C was 2.0 min. Breakdown of phyllanthoside in plasma from other species was slower than in mouse and the initial half-life at 37°C in dog plasma was 30 min, in monkey plasma 33 min and in human plasma 38 min. The same less polar metabolite as in mouse plasma was formed slowly by plasma of monkey and dog. Phyllanthoside did not accumulate in human red blood cells. Binding of phyllanthoside to human plasma protein determined by ultrafiltration at 4°C was 70%.

INTRODUCTION

Phyllanthoside is a glycoside isolated from the roots of the Central American tree *Phyllanthus acuminatus* Vahl [1]. The structure of phyllanthoside has been determined by Pettit et al. [2] and is shown in Fig. 1. Phyllanthoside is an inhibitor of protein synthesis in vitro and when administered intraperitoneally to mice has antitumor activity against intraperitoneal B-16 melanoma and

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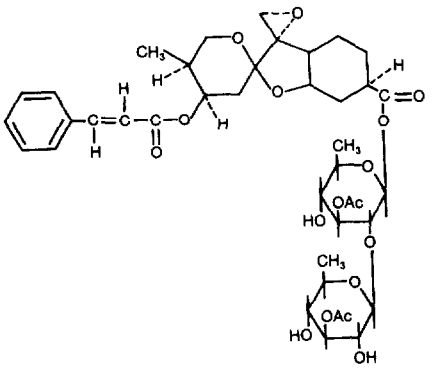


Fig. 1. Structure of phyllanthoside (NSC 328426).

P-388 leukemia [3]. Phyllanthoside is currently being considered by the National Cancer Institute, U.S.A. for eventual clinical trial. We report a sensitive high-performance liquid chromatography (HPLC) assay for phyllanthoside in biological fluids. The assay has been used to show rapid breakdown of phyllanthoside added to plasma of mouse and rat, but much slower breakdown when added to plasma of dog and man.

EXPERIMENTAL

Drugs

Phyllanthoside (NSC 328426) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.). Phyllanthoside was formulated in vials containing 10 mg drug and 100 mg mannitol and reconstituted immediately prior to use with 2 ml of ethanol—Cremophore EL® (5:2.5). 4-Hydroxybiphenyl was obtained from Aldrich (Milwaukee, WI, U.S.A.) and Cremophore EL from Sigma (St. Louis, MO, U.S.A.).

Preparation of samples

To a 0.5-ml volume of plasma were added 1 μg of 4-hydroxybiphenyl as an internal standard, 0.5 ml of 0.1 *M* sodium phosphate buffer, pH 7.0, and 5 ml of ethyl acetate. The mixture was shaken vigorously for 20 min and then centrifuged at 1000 *g* for 10 min at room temperature. The upper ethyl acetate layer was removed and evaporated to dryness under nitrogen at room temperature. The residue was dissolved in 100 μl methanol and 60 μl were taken for HPLC. An alternative extraction procedure for phyllanthoside was used in some of the initial studies. A 0.5-ml volume of plasma containing 1 μg of 4-hydroxybiphenyl was mixed with 0.5 ml of 0.1 *M* sodium phosphate buffer, pH 7.0, and applied to a 1-ml octadecylsilane disposable extraction cartridge (Baker, Phillipsburg, NJ, U.S.A.). The cartridge was washed extensively with water and eluted six times with 1 ml of methanol. The methanol was evaporated to dryness under nitrogen at room temperature and the residue dissolved in 100 μl methanol for HPLC.

High-performance liquid chromatography

The HPLC analysis employed a 25-cm Hibar -II RP-18, 5-μm column (Merck, Darmstadt, F.R.G.) and 50—100% 10-min linear gradient of methanol in 0.3 *M* sodium acetate buffer, pH 4.0, at a flow-rate of 1.5 ml/min using a Hewlett-Packard 1084B liquid chromatograph. Eluting compounds were detected by their absorbance at 270 nm on a Hewlett-Packard 798575A variable-wavelength detector. The output from the detector was fed into a Hewlett-Packard 79850B liquid chromatograph terminal and peak areas were integrated.

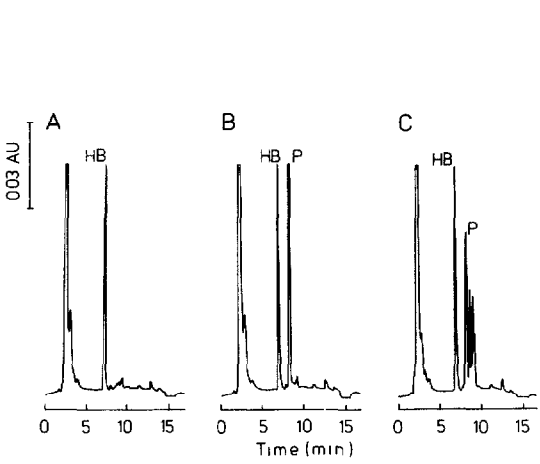
Stability studies

For studies on the stability of phyllanthoside, formulated drug was added to fresh citrate-buffered human blood and plasma at initial concentrations of 16, 5 and 1.6 μg/ml and incubated in the dark at 37°C, 20°C and 4°C for 4 h. Samples (0.5 ml) of plasma, or of plasma prepared from blood after incubation, were taken for analysis of phyllanthoside. For studies on the stability of phyllanthoside in plasma of different species, fresh heparinized plasma was obtained from CDF_1 mouse, Sprague—Dawley rat, mongrel dog, cynomologus macaque monkey and man (male for all species). Phyllanthoside was added at an initial concentration of 25 μg/ml and incubated at 37°C for up to 6 h. Plasma protein binding studies were conducted using phyllanthoside concentrations of 16, 5 and 1.6 μg/ml in human plasma at 4°C, with Centrifree micropartition filters (Amicon, Danvers, MA, U.S.A.). Plasma protein binding was corrected for 9.5% binding of phyllanthoside in 10 m*M* phosphate-buffered 0.9% sodium chloride, pH 7.0 to the filter membrane. Plasma protein binding was also measured by equilibrium dialysis in 1000 vols. 10 m*M* phosphate-buffered 0.9% sodium chloride, pH 7.0 with continual stirring for 20 h at 4°C.

RESULTS

Ethyl acetate extraction provided a simple and reliable means of concentrating phyllanthoside and its metabolites from biological fluids. The efficiency of extraction of phyllanthoside at 1 μg/ml by ethyl acetate was 96% and of 4-hydroxybiphenyl, internal standard, at 2 μg/ml was 95%. An alternative extraction procedure for phyllanthoside was initially adopted using disposable 1-ml octadecylsilane extraction cartridges. The recovery of phyllanthoside by this procedure was 108% and of 4-hydroxybiphenyl, internal standard, was 82%. Further study of cartridge extraction revealed that upon HPLC the peak apparently corresponding to phyllanthoside, in fact had a different retention time (8.04 min) compared to the retention time of authentic phyllanthoside (8.44 min). The identity of the derivative producing the new peak was not established. Ethyl acetate extraction gave an HPLC peak whose retention time corresponded exactly with that of authentic phyllanthoside and ethyl acetate extraction was used for all subsequent studies.

The reversed-phase HPLC procedure gave good separation of phyllanthoside and its breakdown products. A typical chromatogram of phyllanthoside added to human plasma is shown in Fig. 2. The HPLC assay with ethyl acetate extraction was linear up to at least 20 μg/ml and had a lower limit of detectability of 0.25 μg/ml. The coefficient of variation of ten repeated assays was 3.3% at 10 μg/ml, 7.4% at 1 μg/ml and 15.9% at 0.25 μg/ml.



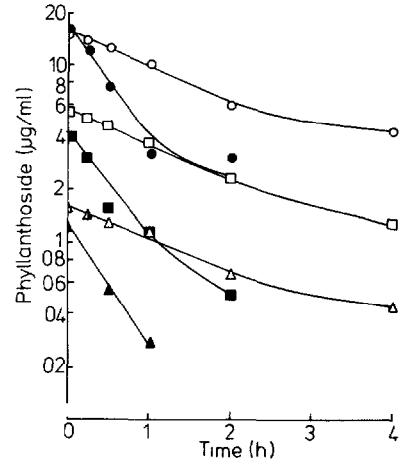


Fig. 2. Chromatogram of phyllanthoside in fresh human plasma. (A) A 0.5-ml volume of blank plasma containing 1 µg of 4-hydroxybiphenyl, internal standard; (B) 0.5 ml plasma containing 16 µg/ml phyllanthoside and 1 µg of 4-hydroxybiphenyl, internal standard; (C) 0.5 ml plasma containing 16 µg/ml phyllanthoside incubated at 37°C for 30 min and 1 µg of 4-hydroxybiphenyl, internal standard. Note the appearance of a number of less polar products. Peaks: HB = 4-hydroxybiphenyl, internal standard; P = phyllanthoside.

Fig. 3. Stability of phyllanthoside in fresh human plasma and blood at 37°C. Phyllanthoside was added at initial concentrations of 16 µg/ml (○, ●), 5 µg/ml (□, ■) and 1.6 µg/ml (△, ▲) to plasma (closed symbols) and blood (open symbols). Values are phyllanthoside concentrations in the plasma.

Having developed an assay procedure for phyllanthoside the stability of the drug was studied under a variety of conditions. Phyllanthoside when formulated at 5 mg/ml with 50 mg/ml mannitol in ethanol—Cremophore EL (5:2.5) showed less than 7% loss of drug over three days when kept in the dark at room temperature, and no detectable loss of drug over three days when kept at 4°C. When phyllanthoside was added to fresh citrate-buffered human blood or plasma biphasic decay curves were observed as shown in Fig. 3. The initial half-life of phyllanthoside in plasma at 37°C was 25 min, at 20°C 74 min and at 4°C 2117 min. The corresponding initial half-lives of plasma phyllanthoside in whole blood were 96, 134 and 1416 min. The mean ratio of the extrapolated zero time concentration of plasma phyllanthoside in blood to the extrapolated zero time concentration in plasma with the same amount of added drug was 1.2. The hematocrit of the blood used for the studies was 46%. These results show that phyllanthoside was not accumulated by red blood cells and that only 61% of the red blood cell volume was available for phyllanthoside distribution. Phyllanthoside was converted by human blood and plasma to a number of less polar derivatives (Fig. 2). The binding of phyllanthoside to human plasma protein determined by ultrafiltration at 4°C was 70% and the binding determined by equilibrium dialysis at 4°C was 32%.

The stability of phyllanthoside at 37°C in fresh heparinized plasma from a number of species was studied (Fig. 4). The initial half-life of phyllanthoside in plasma from dog was 30 min, in plasma from monkey 33 min, and in human plasma 38 min. When added to fresh plasma from mouse and rat the

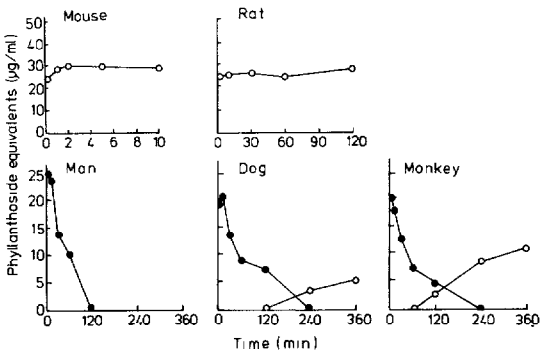


Fig. 4. Stability of phyllanthoside in fresh heparinized plasma of different species. Phyllanthoside (•) was added to the plasma to give an initial concentration of 25 μg/ml and incubated at 37°C. The less polar phyllanthoside metabolite (○) is expressed in phyllanthoside equivalents.

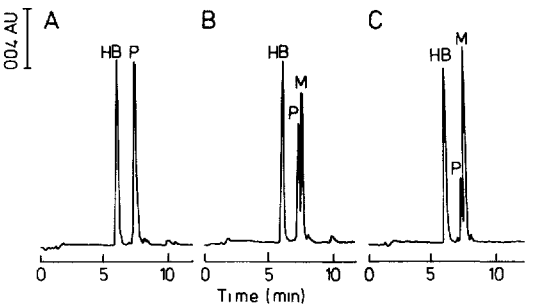


Fig. 5. Chromatograms of phyllanthoside in mouse plasma diluted 1:50 with water. Phyllanthoside was added at an initial concentration of 25 μg/ml and incubated at 37°C. (A) Time zero; (B) 3 min; (C) 5 min. Peaks: HB = 4-hydroxybiphenyl, internal standard, 1 μg/ml; P = phyllanthoside; and M = less polar phyllanthoside metabolite.

disappearance of phyllanthoside was so rapid that no parent drug could be detected even after 15 sec of incubation. Instead, a single less polar metabolite peak was seen. Formation of this metabolite from phyllanthoside was completely inhibited by preheating mouse plasma to 100°C for 10 min. When mouse plasma was diluted with water 1:20 the half-life for disappearance of phyllanthoside was 0.6 min and when diluted with water 1:50 the half-life was 2.0 min (Fig. 5). The same less polar metabolite formed from phyllanthoside by plasma of mouse and rat plasma was formed when phyllanthoside was incubated with plasma of monkey and dog, although much more slowly and the metabolite was not detected until after 1 to 2 h. Human plasma did not appear to form this metabolite in appreciable amounts at any time.

DISCUSSION

Ethyl acetate extraction at neutral pH combined with reversed-phase HPLC and detection at 270 nm provides a sensitive, selective and reproducible assay for phyllanthoside in blood and plasma. The HPLC procedure can separate phyllanthoside from its breakdown products. The breakdown products have

not been identified. Phyllanthoside was relatively slowly degraded by plasma from man, dog and monkey at 37°C, with initial half-lives of 25—38, 30 and 33 min, respectively. The major product formed from phyllanthoside by plasma from dog and monkey was a single less polar compound, but human plasma formed a number of less polar derivatives. When added to mouse and rat plasma phyllanthoside was very rapidly broken down. The speed of the reaction was such that all of the phyllanthoside had been converted to a less polar metabolite, which was apparently the same as that formed by dog and monkey plasma, within 15 sec. This was the time it took to mix the sample and added ethyl acetate to stop the reaction. When mouse plasma was diluted 1:20 with water the half-life of phyllanthoside was 0.6 min and when mouse plasma was diluted 1:50 with water the half-life was 2.0 min. The rapid breakdown of phyllanthoside by mouse plasma could be inhibited by preheating the plasma to 100°C for 10 min, suggesting an enzyme-catalyzed reaction. If the breakdown of phyllanthoside is indeed enzymatic, it appears to be extremely rapid.

Although intraperitoneal phyllanthoside has antitumor activity against intraperitoneal murine B-16 melanoma and intraperitoneal murine P-388 leukemia, intraperitoneal phyllanthoside does not exhibit activity against transplanted murine tumors or human tumor xenografts at other sites in mouse. Furthermore, intravenous phyllanthoside has only marginal activity against intraperitoneal murine B-16 melanoma and murine leukemia P-388 [3]. A possible explanation for these findings could be rapid metabolism of phyllanthoside in the mouse by plasma and possibly at other sites in the body. There is evidence from in vitro cytotoxicity studies that rat hepatocytes convert phyllanthoside to the same less polar metabolite as rat and mouse plasma, with a decrease in cytotoxicity to cultured A204 human rhabdomyosarcoma cells [4]. Whether the antitumor activity of phyllanthoside, or even toxicity to the host, is the same in mouse and in other species such as dog, monkey or man, where plasma does not degrade phyllanthoside as rapidly, is not known.

In summary, a sensitive and selective HPLC assay for the antitumor glycoside phyllanthoside in biological fluids has been developed. Phyllanthoside is converted to a less polar derivative relatively slowly by plasma from dog and monkey, but very rapidly by plasma from mouse and rat. Human plasma breaks down phyllanthoside relatively slowly to a number of less polar metabolites.

ACKNOWLEDGEMENT

This work was supported by NCI Contract N01-CM-37601.

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