Liquid-Chromatographic Determination of Dolichols in Urine

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A reversed-phase “high-performance” liquid chromatographic assay for dolichols-18, -19 and -20 in urine is described. Dolichols are extracted from urine by using C18 cartridges and are chromatographed with a mobile phase consisting of 2-propanol/methanol, the effluent being monitored at 210 nm. The useful lower limit of sensitivity for quantification is 4 pmol (5 ng) of each dolichol per 5-µL injection, corresponding to 1.6 nmol (2 ng) per liter of urine. Heneicosaprenol is satisfactory as the internal standard. Peak heights and the amounts of dolichols applied to the column are linearly related from 4 to 110 pmol. Mean analytical recovery was 71%. For three different concentrations the mean within-assay CV was 6.4%, the between-assay CV 11%. The normal reference interval of total dolichols found for healthy adults was 17–101 µg/24 h (n = 30) or 1.9–11 µg per millimole of creatinine (n = 39). I determined the distribution of the main dolichols in urine and applied the assay also for samples from alcoholics.

Additional Keyphrases: reference interval · alcoholism · polyprenols · cancer · Alzheimer’s disease · aging · nervous-system disorders

Dolichols are long-chain polyprenols with the general structure of \(\text{H}(\text{CH}_2\text{CH}_2\text{CH}_3)=\text{CH}_2\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{OH}\) containing 16 to 23 isoprene units. They exist in tissues and biological fluids as free alcohols, phosphate esters, and esters of fatty acids (1). The only known function of these compounds is that of dolichol phosphates as lipid carriers for saccharide residues in the synthesis of asparagus-linked glycoproteins (2). Except for protein glycosylation, relatively little is known about the physiological role and catabolism of dolichols in health and disease. It has been suggested that alcohol dehydrogenase is involved in the oxidation of dolichols (3). Increased content of dolichols in brain and urine sediments has been found thus far in aging (4), in ceroid lipofuscinoses (5), and in Alzheimer’s disease (6). Also, chronic alcoholics excrete high amounts of dolichols (3), as do newborns whose mothers are heavy alcohol users (7).

Previously described methods for dolichols are tedious, involving two to four solvent-extraction steps (8, 9) or isolation by thin-layer chromatography (3, 4). Although liquid chromatography (HPLC) has been applied for dolichol determinations (3, 4–10), these, except for one synthesized in the authors’ laboratory (9), did not include an internal standard. As an alternative, therefore, I developed the present rapid reversed-phase HPLC assay for urinary dolichols in which they are first extracted from urine onto solid-phase extraction columns, with heneicosaprenol as internal standard. Dolichols of different chain length are well resolved from the internal standard and interfering peaks.

Materials and Methods

Apparatus. The chromatographic system consisted of a Varian 5000 liquid chromatograph combined with a Vistas 402 chromatographic data system (Varian Associates, Walnut Creek, CA) and a Perkin-Elmer recorder 056-1002 (Hitachi Ltd., Tokyo, Japan). I used a 250 × 4.6 mm reversed-phase C18 column (Spherisorb S5 ODS2, Phase Separations Ltd., Queensferry, Clwyd, U.K.). Sep-pak C18 cartridges were from Waters Associates, Milford, MA.

Reagents. Dolichol-18, -19, and -20 and heneicosaprenol were from Sigma Chemical Co., St. Louis, MO. Stock 0.5 g/L solutions in 2-propanol/methanol (72/28 by vol.) were stored at −20°C and diluted to 10 mg/L before use. “HPLC-grade” methanol and 2-propanol were from Rathburn Chemicals Ltd., Walkerburn, Scotland. Solvents and all other chemicals were analytical grade.

Sample treatment. Collect 24-h urine specimens or untiimed urine samples. If these are not to be promptly assessed, store them at −20°C. To 20 mL of whole-mixed urine sample add 40 µL of 10 mg/L heneicosaprenol solution as internal standard. Mix well and apply to a C18 Sep-pak column equilibrated with 10 mL of methanol followed by 10 mL of water. After washing with 10 mL of water and 10 mL of methanol, elute dolichols with 6 mL of ethanol/methanol/2-propanol (90/5/5 by vol.). Evaporate the collected eluates under nitrogen and dissolve the residues in 40 µL of 2-propanol/methanol (72/28).

Chromatographic conditions. Inject 10 µL of the 2-propanol/methanol solution into the chromatographic system. Use a flow rate of 1.0 mL/min with a mobile phase consisting of 2-propanol/methanol (72/28 by vol.) at room temperature. The detection wavelength is 210 nm, detector range 0.01 A full scale, and recorder chart speed 5 mm/min. With the Vistas 402 data system I used, the attenuator setting was 4, corresponding to 0.008 A full scale.

Analytical variables. The calibration curve was obtained with each dolichol dissolved in the mobile phase, 1 or 10 mg/L. To establish the linearity of the detector response to dolichols, I injected standard dolichols in amounts of 5 to 150 ng (4 to 120 pmol). The calibration curves were obtained by plotting the peak heights of individual dolichols vs the amounts injected.

Analytical recovery was evaluated by adding 50 to 150 ng of each dolichol to 20 mL of urine and asaying. The samples were taken through the whole procedure and results compared to the amounts obtained from calibration curves without the internal standard.

To test reproducibility, I used urine from control subjects containing 10 to 92 µg of dolichols per liter.

Urinary creatinine was determined by the kinetic picrate method (11), with the Hitachi 705 analyzer.
Results

Analytical Variables

Linearity and sensitivity. Linear calibration curves for peak heights (y) vs quantities of dolichols (x) were obtained:

\[ y = 1.65x - 0.40, \quad r = 0.9998 \text{ for dolichol-18}; \quad y = 1.10x + 0.11, \quad r = 0.9998 \text{ for dolichol-19}; \quad y = 2.16x - 0.44, \quad r = 0.999 \text{ for dolichol-20} \quad (n = 8). \]

The linear ranges were from 5.0 to 150 ng (4.0 to 120 pmol) per 5-μL injection for each dolichol (Figure 1). The minimum detectable amount of each dolichol was 5 ng (4 pmol) when the detector was set at 0.01 A full-scale. This corresponds to 2 μg (1.6 nmol) of each dolichol or 6 μg (4.8 nmol) of total dolichol per liter of urine.

Analytical recovery. Mean analytical recoveries of dolichols-18, -19, and -20 from normal urine supplemented with 50 to 150 ng (40 to 120 pmol) of these compounds per 20 mL of urine were 65, 79, and 68%, respectively (n = 5, range 52 to 91%), with an overall mean recovery of 71%.

Precision. As shown in Table 1, the within-assay CV, calculated from values for three samples, ranged from 6.0 to 6.8%, the between-assay CVs from 7.6 to 14%.

Analysis of Urine Samples

Separation of dolichols-18, -19, and -20. The urines, 24-h collections and untimed samples, from healthy subjects among the hospital personnel and medical students were analyzed. Untimed urine specimens were obtained also from chronic alcoholics. Figure 2 shows representative chromatograms for a standard mixture of dolichols and the internal standard (A), for dolichols in normal urine (B) and with the internal standard (C), and for dolichols in an alcoholic’s urine (D). In these, dolichols-18, -19, and -20 were eluted as sharp, symmetrical peaks at 10.2–10.4 min, 11.7–11.9 min and 13.5–13.7 min, respectively. Elution time for the internal standard, heneicosaprenol, was 15.1–15.4 min. In all cases dolichols and heneicosaprenol were well resolved from unidentified material eluting near the solvent front. Dolichols were identified by comparison of retention times and by co-elution with standards.

The major dolichol homologs in untimed urines from healthy subjects (n = 30) were dolichols-18, -19, and -20 in the proportions of 15, 67, and 18%, respectively. The dolichol distribution in 24-h urines from the same individuals remained unchanged: 15, 68, and 17%.

Urinary total dolichol excretion. I measured the excretion of total dolichols by healthy subjects and alcoholics. The total dolichols excreted per 24 h by each of 27 healthy persons ranged from 23.4 to 80.4 μg (mean 53.8 μg, SD 14.4). The reference interval (mean ± 2 SD) for the 24-h total dolichol excretion was 25 to 85 μg. The dolichols were analyzed also in untimed urines from the same subjects and medical students (n = 39) and expressed as micrograms of dolichol per millimole of urinary creatinine. They ranged from 2.6 to 12 μg per mmol of creatinine (mean 6.7, SD 2.4).

The correlation between the 24-h dolichol excretion and dolichols calculated per millimole of urinary creatinine in random samples was determined in 26 healthy subjects. A good correlation (r = 0.82) was obtained, as shown in Figure 4.

Discussion

The aim of the present study was to develop a rapid, practical method for determination of total dolichols in human urine by use of HPLC. Measurements of dolichol excretion have been found useful in diagnosis of three types of neuronal ceroid lipofuscinosis where significant increase of free dolichols has been detected in urinary sediments (5, 8). These findings have been, however, disputed by Bennett et al. (10). In cancer patients, urinary dolichols have been reported to be five to 40 times the normal values, suggesting a metabolic abnormality of dolichol in patients with metastatic cancer (12). High dolichol concentrations also have been measured in human cerebral cortex of patients with Alzheimer's disease (13) and neuronal ceroid lipofuscinosis (14), as well in brains of elderly (4).

All recent assay methods are rather time-consuming, involving either several solvent-extraction steps (5, 8) or thin-layer chromatography (4) before quantification by HPLC. I developed the present method by using C18-cartridges for sample preparation, which greatly simplifies the determination. Heneicosaprenol can be chosen as the internal standard, because no co-eluting peaks are found in urine (Figure 2B). In the present method the minimal detectable concentration of 2 μg (1.6 nmol) of each dolichol per liter is adequate.

The good correlation (r = 0.82) between dolichols in 24-h collections and in random specimens (Figure 4) makes it possible to use untimed urine specimens for the assay. This is an advantage because specimen collection is simpler.

The mean of the control group (n = 39), 6.7 μg per millimole of creatinine, is lower than that of Bennett et al. (10), who found about 27 μg/mmol. Their value is the mean for only 10 subjects, obtained without use of an internal standard, which may partly explain the difference. Total dolichols have been previously quantified also per milligram.
Fig. 2. Chromatograms of dolichols in (A) a standard mixture with the internal standard (IS), urine without (B) and with (C) the internal standard added, and (D) urine from an alcoholic. The numbers indicate time of elution (minutes since sample injection). Peaks: 1, dolichol-18; 2, dolichol-19; 3, dolichol-20.
The dolichols-18, Figure 3. Urinary dolichols in healthy subjects (A) and alcoholics (B)
The ranges (and means) of dolichols are 2.5-12 (6.7) μg/mmol creatinine for healthy subjects (A) and 4.7-80 (23) μg/mmol creatinine for alcoholics (B)

of total lipid (5, 8), which is a rather tedious procedure requiring also the lipid assay.

My results show that the percentage distribution of dolichols-18, -19, and -20 in urine is constant. For untimed as well as for 24-h urine specimens the distributions are similar, with dolichol-19 as the main component (68%). Pullarkat and Reha (4) have reported that dolichol-19 also predominates in grey and white matter of human brain.

The percentage of 2-propanol in the mobile phase greatly affects the retention of dolichols. If the separation is not satisfactory the content of 2-propanol in the mobile phase can be decreased to achieve the best separation of dolichols.

In summary, the present investigation shows that urinary dolichols can be rapidly and precisely determined by HPLC after extraction on the reversed-phase sorbent. The method is convenient enough for routine use during clinical trials. My preliminary results show that alcoholics excrete high amounts of dolichols in urine. All patients had histories of heavy alcohol use. But because increased urinary dolichol is not specific for chronic alcoholism (4, 5, 8, 12, 13), further studies are warranted to establish the diagnostic value of dolichols as markers of alcohol intake.

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References