Journal of Chromatography, 343 (1985) 413—418
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2694

Note

Quantitation of tissue levels of dolichyl pyrophosphate by high-performance liquid chromatography on silica

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(Received April 2nd, 1985)

Dolichyl pyrophosphate (Dol-P-P) participates in the dolichol-mediated pathway of N-linked glycoprotein synthesis and is a possible intermediate in the biosynthetic pathway of dolichyl phosphate (Dol-P) [1, 2]. To date, no straightforward assay is available for the analysis of Dol-P-P, and thus investigations concerning the metabolism of this important phospholipid have been severely limited. In the present paper, we describe a high-performance liquid chromatographic (HPLC) procedure for the quantitation of Dol-P-P on non-bonded silica. The procedure was also found to be applicable to the analysis of solanesyl pyrophosphate (Sol-P-P), the long-chain all-*trans*-polyisoprenoid which serves as the donor for the side-chain of ubiquinone. In addition, we have developed a rapid procedure for the extraction and partial purification of Dol-P-P from tissue. By using the HPLC technique for quantitation, we were able to determine directly, for the first time, the levels of free and total Dol-P-P in rat liver.

EXPERIMENTAL

Solanesol and pig liver dolichol were from Sigma. Triethylamine and trichloroacetonitrile were from Aldrich. Both Dol-P-P and Sol-P-P were made according to the following procedure. A 15-mg amount of the corresponding alcohol was taken to dryness. After the addition of 0.55 ml of trichloroacetonitrile, 18 ml of a solution of 0.154 *M* triethylammonium phosphate (prepared by the addition of 271 mg phosphoric acid and 780 μl triethylamine to 18 ml

0378-4347/85/$03.30

acetonitrile) were added in nine 2-ml aliquots 30 min apart. The reaction was allowed to stand overnight prior to the addition of 22 ml chloroform—methanol (2:1) and 20 ml water. After vortexing and centrifugation, the lower phase was applied to a 5-ml column of DEAE-cellulose equilibrated in chloroform—methanol (1:1). After washing the column with 20 ml of the same solvent, the product was eluted with 20 ml chloroform—methanol (1:1) containing 0.3 *M* ammonium acetate. The sample was then treated with water and chloroform to form two phases, vortexed and centrifuged.

[^{3}H]Dol-P-P was prepared from [^{3}H]dolichol (1.5 mCi, 1.5 mg) using the same procedure as above scaled down ten-fold. [^{3}H]Dolichol was prepared according to Keenan and Kruczek [3]. [β-^{32}P]Dol-P-P was prepared using Dol-P as starting material. Dol-P (0.55 μmol) was taken to dryness in a 100 X 13 mm screw-capped tube, followed by the addition of 25 μl dichloroethane, 5 μl of a 60 mg/ml solution of triethylamine in dichloroethane, and 30 μl of 13 mg/ml diphenylchlorophosphate in dichloroethane. After standing 1 h at room temperature, the sample was treated with 2 ml chloroform—methanol (2:1) and 0.5 ml water. After vortexing and centrifugation, the lower phase was applied to a 2-ml DEAE-cellulose column equilibrated in chloroform—methanol (2:1). The column was washed with 10 ml of the same solvent prior to elution of the diphenylphosphochloridate derivative with 10 ml of chloroform—methanol (2:1) containing 25 m*M* ammonium acetate. The eluted sample was treated with 0.25 vol. water and centrifuged. The lower phase was washed with 50% methanol, evaporated to dryness at room temperature and dissolved in 50 μl of dichloroethane containing 10% pyridine. The sample was then phosphorylated with 60 μl of a [^{32}P]triethylammonium phosphate (TEAP) solution. The TEAP was prepared as follows: 1 μmol phosphoric acid (Fluka) was taken to dryness with 0.25 mCi $H_3{}^{32}PO_4$ (ICN), and then treated with three portions of 100 μl toluene with drying under argon after each addition. To the dried sample were added 20 μl of 0.2 *M* triethylamine in acetonitrile and 40 μl acetonitrile to form [^{32}P]TEAP. This entire solution was added to the derivative and incubated overnight at room temperature. The sample was then treated with 2 ml chloroform—methanol (2:1), 0.5 ml water, vortexed and centrifuged. The lower phase, containing the [β-^{32}P]Dol-P-P, was applied to DEAE-cellulose and the product isolated as described above.

Extraction and purification of Sol-P-P and free and total Dol-P-P

Rat liver was homogenized in 20 vols. chloroform—methanol—80 m*M* phosphoric acid (10:10:3) and filtered over glass fiber filters. When total Dol-P-P was to be analyzed, the extract was first treated with hydrochloric acid (0.065 *M* hydrochloric acid final concentration), and incubated for 30 min at 50°C. Control experiments demonstrated that Dol-P-P-[^{3}H]N-acetylglucosamine was > 85% hydrolyzed under these conditions while [β-^{32}P]Dol-P-P remained intact. The acidified extract was then adjusted to a final concentration (corrected for neutralization) of 0.1 *M* sodium hydroxide and incubated for 15 min at 37°C. Control experiments showed that this treatment quantitatively degrades all the major phospholipids [as assessed by thin-layer chromatographic (TLC) analysis and phosphate spray] without significant hydrolysis of Dol-P-P.

For the analysis of Sol-P-P and free Dol-P-P, extracts were treated as above except that the acid hydrolysis step was omitted. Following treatment, 0.4 vol. of chloroform and 0.2 vol. water, were added and the samples vortexed and centrifuged to form two phases. The lower phase was applied directly to a 2-ml column of DEAE-cellulose equilibrated with chloroform–methanol (2:1). The column was washed with 10 ml of 2:1, and then 10 ml of chloroform–acetic acid (3:1) prior to elution of Dol-P-P and Sol-P-P with chloroform–methanol (2:1) containing 0.3 *M* ammonium acetate. The eluted sample was treated with 0.4 vol. chloroform, 0.2 vol. water, vortexed and centrifuged. The lower phase was transferred to a fresh tube, evaporated to dryness under a stream of argon at 50°C and dissolved in 100 μl of the mobile phase. A 50-μl aliquot was injected into a 100-μl sample loop in the liquid chromatograph. Either [^{3}H]Dol-P-P or [^{32}P]Dol-P-P was added (ca. 10 000 cpm freshly prepared and purified by HPLC) to the original sample to correct for losses. The Dol-P-P present in the original sample was calculated from the formula:

$$\frac{[^3\text{H}]\text{Dol-P-P added to original sample}}{[^3\text{H}]\text{Dol-P-P recovered from HPLC}} \times \frac{\text{integration units of Dol-P-P in sample peak}}{\text{integration units per } \mu\text{g standard Dol-P-P eluted from HPLC}}$$

High-performance liquid chromatography

HPLC was carried out on a Laboratory Data Control Constametric II chromatograph equipped with a variable-wavelength detector set at 210 nm. UV data were analyzed using a Shimadzu C-R3A Chromatopac. Adsorption chromatography was performed on 22 × 0.46 cm Brownlee Spheri-5 columns coupled to 3-cm guard cartridges. The mobile phase, consisting of hexane–isopropanol–50% aqueous phosphoric acid (950:55:1), was pumped at a flow-rate of either 1.5 or 2.5 ml/min for Dol-P-P and 2.5 ml/min for Sol-P-P (see figure legends). For removal of orthophosphoric acid from samples collected from HPLC, 1.5 vols. of isopropanol–water (3:2) were added followed by centrifugation. The upper phase contained the phosphoric acid-free Dol-P-P or Sol-P-P. Fresh, unused columns were treated according to the method of Keller et al. [4] prior to equilibration with the mobile phase.

RESULTS AND DISCUSSION

The development of a silica HPLC technique for Dol-P-P analysis was based on the work of Yandrasitz et al. [5], who showed that inclusion of ion-suppressing acid in a mobile phase of hexane–isopropanol resulted in sharp, well resolved peaks of the common phospholipids. We have recently reported [4, 6] on the silica HPLC of Dol-P using ion-suppression with phosphoric acid in a mobile phase consisting of hexane–isopropanol–1.4 *M* phosphoric acid (965:35:0.5). For Dol-P-P, we found that substantially greater concentrations of phosphoric acid were required for elution, presumably due to the fact that Dol-P-P has one more negative charge than Dol-P. The mobile phase finally adopted (hexane–isopropanol–50% phosphoric acid, 950:55:1) elutes Dol-P-P at approximately 7 min at a flow-rate of 2.5 ml/min (Fig. 1a). Interestingly, increasing the concentration of the polar isopropanol increases the elution

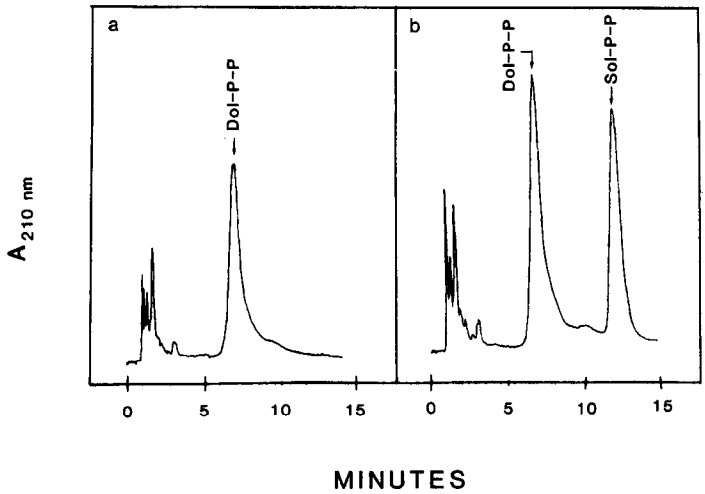

Fig. 1. HPLC of standard. (a) Dol-P-P (1.7 μg); (b) Dol-P-P (1.7 μg) and Sol-P-P (1.5 μg). The flow-rate was 2.5 ml/min. For details, see Experimental.

time, an observation also made in the original work of Yandrasitz et al. [5] on the major phospholipids. A 1-μg amount of solute yields a peak of approximately 50% full scale deflection with the UV monitor on 0.06 a.u.f.s. Recovery, based on elution of [^{3}H]Dol-P-P standard (prepurified by HPLC) averaged 85% (three runs). The system developed for Dol-P-P was also found to be suitable for Sol-P-P (Fig. 1b), the precursor to the side-chain of ubiquinone.

To determine if the HPLC technique could be used to satisfactorily quantitate tissue levels, we developed a rapid procedure for the extraction of free and total Dol-P-P (see Experimental). Fig. 2 shows a typical HPLC profile of samples prepared from rat liver and analyzed for free Dol-P-P (Fig. 2a) and total Dol-P-P (Fig. 2b). As shown, a far greater amount was found in the sample treated with acid (total Dol-P-P) indicating a substantial percentage of the Dol-P-P in liver is derivatized by acid labile bonds, probably in the form of oligosaccharide chains [1]. We also attempted to determine the levels of Sol-P-P in rat liver. Because the α-unsaturated isoprene renders this compound acid labile, we carried out only the mild base treatment on the chloroform–methanol–80 m*M* phosphoric acid (10:10:3) extract prior to DEAE-cellulose chromatography (see Experimental). To ensure that the extraction and purification scheme resulted in the recovery of Sol-P-P we spiked one extract with 5 μg of standard material. As shown in Fig. 2c, we were able to demonstrate recovery (10% overall) from this spiked sample; however, there was no detectable solanesyl pyrophosphate in the unspiked sample. From this data we conclude that the level of solanesyl pyrophosphate in rat liver is less than 1 μg/g wet weight.

From the data of Fig. 2a and b, we concluded that the extraction and HPLC procedure could be used to accurately determine free and total Dol-P-P levels. We therefore carried out analysis on eight different rat liver samples. In addition, and as a check that the livers were representative of samples previously analyzed, we carried out total Dol-P analysis using an HPLC procedure previously developed in this laboratory [4]. The results given in Table I show

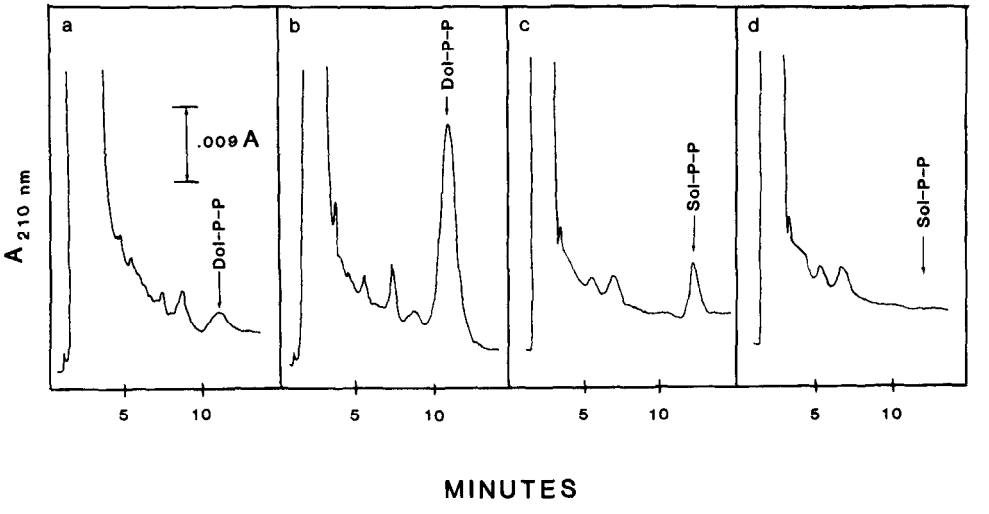

Fig. 2. HPLC analysis of prenyl pyrophosphates derived from 2 g of rat liver. (a) Free Dol-P-P; (b) total Dol-P-P; (c) sample spiked with 5 μg Sol-P-P; (d) unspiked sample. Flow-rate in a and b: 1.5 ml/min; flow-rate in c and d: 2.5 ml/min.

TABLE I

LEVELS OF FREE Dol-P-P, TOTAL Dol-P-P, TOTAL Dol-P AND Sol-P-P IN RAT LIVER

Compound	Number of animals	Level (μg/g wet wt. ± S.D.)
Dol-P	5	17.5 ± 3.9
Free Dol-P-P	8	0.4 ± 0.2
Total Dol-P-P	8	4.7 ± 1.2
Sol-P-P	2	<1.0

approximately twelve times more total Dol-P-P than free Dol-P-P in rat liver. Analysis of total Dol-P gave 17.6 μg/g, in reasonable agreement with our previously determined level of 14.7 μg/g [5].

The development of an HPLC system for the analysis of Dol-P-P from tissue samples will now allow the determination of Dol-P-P levels from a wide variety of organs and tissues. In addition, the HPLC technique will permit radiochemical analysis of Dol-P-P and Sol-P-P generated by in vitro incubations with radiolabelled precursors (e.g. [^{14}C] isopentenyl pyrophosphate and [^{3}H] mevalonolactone).

ACKNOWLEDGEMENTS

This work was supported by Grants GM 25364 and CA 28781 from the National Institutes of Health. R.K.K. and W.L.A. are recipients of Research Career Development Awards from the NIH. We thank Margaret Kuligofski for word processing assistance.

REFERENCES

1 W.J. Lennarz, CRC Crit. Rev. Biochem., 14 (1983) 257.
2 M.G. Scher and C.J. Waechter, J. Biol. Chem., 259 (1984) 14580.
3 R.W. Keenan and M. Kruczek, Anal. Biochem., 69 (1975) 504.
4 R.K. Keller, M. Fuller, G.D. Rottler and L.W. Connelly, Anal. Biochem., 147 (1985) 166
5 J.R. Yandrasitz, G. Berry and S. Segal, J. Chromatogr., 225 (1981) 319.
6 W.L. Adair and R.K. Keller, Methods Enzymol., 111 (1985) 201.