Journal of Chromatography, 177 (1979) 368–371
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CHROM. 12,032

Note

Rapid separation of *p*-nitrophenol and its glucuronide and sulfate conjugates by reversed-phase high-performance liquid chromatography

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(Received May 15th, 1979)

p-Nitrophenol (PNP) is excreted entirely as its glucuronide and sulfate conjugates in both mammals and birds[1–3]. PNP can easily be detected and quantified in biological samples by virtue of its strong visable absorption at 390 nm. These properties make PNP an excellent substrate for the study of phenolic conjugation enzymes. Such studies require not only the determination of PNP but also of its conjugates for which few methods are available. Thin-layer chromatography on silica gel has been used to separate PNP from its glucuronide and sulfate conjugates; however, quantification of the separated compounds requires time consuming elution procedures[3,4]. An anion-exchange high-performance liquid chromatographic separation of PNP conjugates has been reported[5] but in this system the glucuronide is eluted with the column void volume making this method unsuitable for the quantitative analysis of the glucuronide in biological samples. In addition, the elution of the sulfate requires the use of buffers of extremely high ionic strength (1 *M* KCl) and results in poor peak shape.

In the course of an investigation into the renal conjugation and elimination of phenols, it became necessary to develop a sensitive method for the determination of plasma and urine levels of PNP and its glucuronide and sulfate conjugates. This communication describes the conditions for the simultaneous analysis of PNP and its conjugates in biological samples using reversed-phase high-performance liquid chromatography.

EXPERIMENTAL

Chemicals

PNP and *p*-nitrophenyl-β-D-glucuronide (PNP-G) were obtained from Sigma (St. Louis, Mo., U.S.A.). *p*-Nitrophenyl sulfate (PNP-S) was obtained from Boehringer (Indianapolis, Ind., U.S.A.).

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A pump was used. Samples were injected through a 20-μl loop injector valve (Rheodyne Model 7120) onto a 30 cm × 3.9 mm I.D. μBondapak C_{18} column (Waters Assoc., 10 μm average particle size). The eluted compounds were detected by a dual wavelength ultraviolet (UV) photometer (Altex Model 152) operating at 280 nm.

Chromatographic conditions

The mobile phase consisted of 10 m*M* potassium phosphate, pH 2.75, containing 20% acetonitrile. The column was operated at ambient temperature. The flow-rate was 1.0 ml/min with an inlet pressure of approximately 1200 p.s.i.g.

Sample preparation

Standards were prepared in distilled water. Rat urine was diluted in distilled water and injected onto the column without further preparation. Rat plasma was prepared in the following way. A 50-μl plasma sample was vortexed in 200 μl of methanol. The resulting precipitate was pelleted by centrifugation and the supernatant containing the PNP and its conjugates was collected. The pellet was then resuspended in 200 μl of fresh methanol, centrifuged and the supernatant collected. The combined supernatants were evaporated to dryness under nitrogen and reconstituted with distilled water.

RESULTS AND DISCUSSION

The mobile phase consisting of 10 m*M* K_2HPO_4, pH 2.75, containing 20% acetonitrile provided excellent retention and separation of PNP and its glucuronide

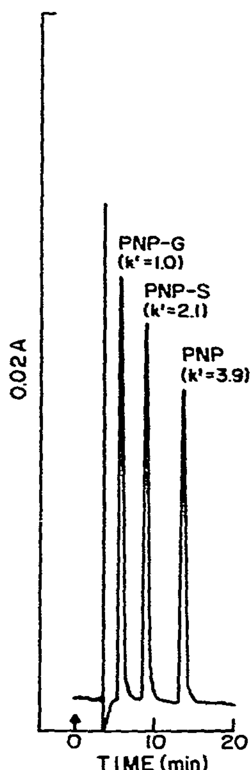

Fig. 1. Chromatogram of PNP (1.0 nmole), PNP-S (0.6 nmole) and PNP-G (0.6 nmole). Eluent: 10 m*M* K_2HPO_4, pH 2.75, containing 20% acetonitrile; column temperature: ambient; flow-rate: 1.0 ml/min; inlet pressure: 1200 p.s.i.g. The constant, k', is defined as: retention volume − column void volume/column void volume.

and sulfate conjugates (Fig. 1). Buffers of pH greater than 3.0 resulted in inadequate retention of the glucuronide. Eluents containing organic solvents of greater and lower polarity than acetonitrile were also tried. However, methanol at several concentrations, failed to resolve the glucuronide from other UV absorbing substances in the biological samples and, in addition, poor peak shape was observed. Solvents containing *n*-propanol also gave poor resolution of the glucuronide.

The methods described above have been applied to the determination of PNP conjugates in rat plasma and urine following an infusion of PNP into the femoral vein (Fig. 2). Analysis time for the determination of all three compounds was less than 16 min. Methanol extraction of the plasma produced sufficient deproteinization to allow repeated injections of the reconstituted extracts without contamination of the column. The extraction procedure yielded recoveries greater than 98% for PNP and greater than 95% for the conjugates.

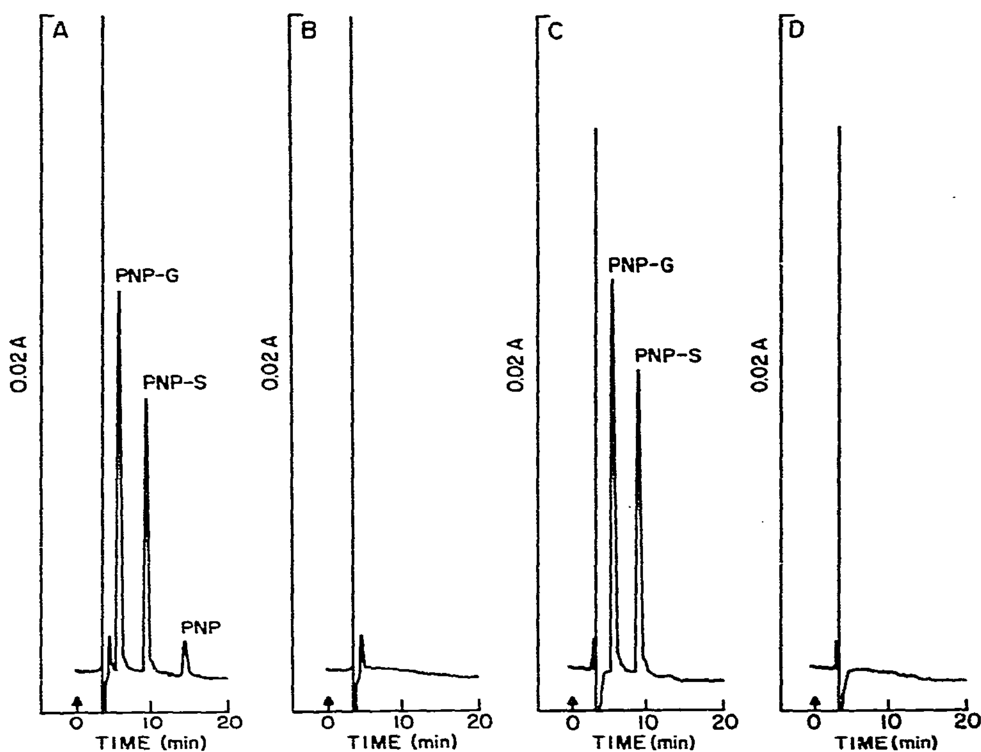

Fig. 2. Chromatograms of PNP, PNP-G and PNP-S in rat plasma and urine. Control plasma and urine samples were collected prior to the start of an intravenous infusion of PNP (2.0 μmole/min/kg). Chromatograms of control plasma (B) and control urine (D) are shown. (A) and (C) show chromatograms of plasma and urine, respectively, collected at 165 min after the start of the PNP infusion. Eluent: 10 m*M* K_2HPO_4, pH 2.75, containing 20% acetonitrile; column temperature: ambient; flow-rate: 1.0 ml/min; inlet pressure: 1200 p.s.i.g.

In summary, a rapid and sensitive method has been developed for the simultaneous determination of PNP and its glucuronide and sulfate conjugates in biological

samples. Since PNP is an excellent substrate for phenol sulfotransferase and glucuronyl-transferase, this method provides a convenient analytical method for both the *in vivo* and *in vitro* assay of these enzymes.

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