Journal of Chromatography, 195 (1980) 431–434
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,829

Note

High-performance liquid chromatography of glucuronide and sulfate conjugates using ion-pair chromatography

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(First received November 5th, 1979; revised manuscript received March 11th, 1980)

Few methods are available for qualitative and quantitative analysis of sulfate and glucuronide conjugates of drugs and chemicals metabolized by the body. The wide range of polarities of a parent molecule, a biological metabolite and of highly polar conjugates makes the use of a single chromatographic separation difficult. Some methods presently employed to separate conjugates use ion-exchange chromatography[1], ion-exclusion–partition chromatography[2], reversed-phase chromatography[3], counter-current liquid–liquid partition[4], and gas chromatography after chemical derivatization[5]. Ion-pair reversed-phase high-performance liquid chromatography (HPLC) has been used on some steroid conjugates and some organic sulfates and carboxylic acids[6,7].

For the purpose of developing a single separation of all species found after metabolism, ion-pair reversed-phase HPLC has been examined. The system was tested for its ability to separate the normal mixture found after metabolism. This consists of a separation of glucuronide and sulfate conjugates of the same substance from each other and from the unconjugated material (precursor). The system was also examined for its ability to separate glucuronide (or sulfate) conjugates of different substances.

EXPERIMENTAL

Chemicals

p-Nitrophenyl glucuronide, *p*-nitrophenyl sulfate, α-naphthyl glucuronide, α-naphthyl sulfate, phenolphthalein glucuronide, *p*-acetylphenyl sulfate, and *p*-nitrocatechol sulfate were used as supplied by Sigma (St. Louis, MO, U.S.A.). Tetrabutylammonium bromide and *p*-nitrophenol were obtained from Eastman-Kodak (Rochester, NY, U.S.A.), and α-naphthol was purchased from Matheson, Coleman & Bell (Norwood, OH, U.S.A.). Harmol glucuronide and harmol sulfate were obtained as a generous gift from Dr. Klaus Brendel (Pharmacology Department, University of Arizona). The solvents used were filtered through a 5.0-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) and de-gassed before use. Samples for analysis were prepared as a 0.02% solution in water adjusted to pH 6–8 and the ion-pairing reagent, tetrabutylammonium bromide, was prepared as a 0.01 *M* solution in water and in methanol.

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Apparatus

A Spectra-Physics Model 3500 high-performance liquid chromatograph equipped with an ultraviolet detector at wavelength 280 nm was employed and the column was a Spectra-Physics 250 × 3 mm I.D. stainless-steel column packed with 10-μm Spherisorb ODS (octadecylsilane, C_{18}). The injection port volume was 10 μl.

Procedure

Isocratic solvent compositions between 30 and 50% methanol in water were required for separation. The system was flushed with 100% methanol at the conclusion of each day and columns were occasionally cleaned by purging the system successively with methanol, ethyl acetate, chloroform and heptane and then reversing the series. A solvent flow-rate of 0.8 ml/min was used in the experiments.

RESULTS AND DISCUSSION

Ion-pair reversed-phase HPLC, using isocratic solvent compositions, was found to be sufficient to cleanly separate the compounds studied. Solvent compositions between 30% and 50% methanol in water using the HPLC conditions described above resulted in separations of *p*-nitrophenol from its corresponding glucuronide and sulfate conjugates and of α-naphthol from its corresponding glucuronide and sulfate conjugates (Table I). This separation models the mixture of compounds that might be found in excreta after administration of a chemical or drug. All of the naphthol species should separate in the form of ion-pairs at pH 6–8 as the pK_a of α-naphthol is 3.7 (ref. 8) and the conjugates should be stronger acids than the parent compound[9]. *p*-Nitrophenol may be only partly ionized as its pK_a is 7.15 (ref. 8) and the pH of the water was not adjusted with any greater precision than 6–8.

TABLE I

CAPACITY FACTORS OF *p*-NITROPHENOL AND α-NAPHTHOL AND CONJUGATES IN DIFFERENT SOLVENT COMPOSITIONS

Compound	*Capacity factor*				
	Methanol (%)				
	50	*45*	*40*	*35*	*30*
p-Nitrophenyl glucuronide	0.75	1.1	1.4	1.8	4.9
p-Nitrophenyl sulfate	2.0	3.1	5.0	10.9	25.5
p-Nitrophenol	1.4	2.1	3.2	5.6	6.6
α-Naphthyl glucuronide	1.2	2.4	4.8	7.1	23.2
α-Naphthyl sulfate	2.8	5.8	12.2	33.0	78.5
α-Naphthol	3.4	5.8	10.1	17.8	27.6

Four glucuronide conjugates (Fig. 1) and four sulfate conjugates (Fig. 2) could be separated from one another demonstrating that the separation was dependent on the parent molecule as well as the conjugating moiety. The sulfate conjugates were structurally similar to one another and the method was selective enough to separate the mixture. These results are similar to the work of Wahlund and co-workers[6,7].

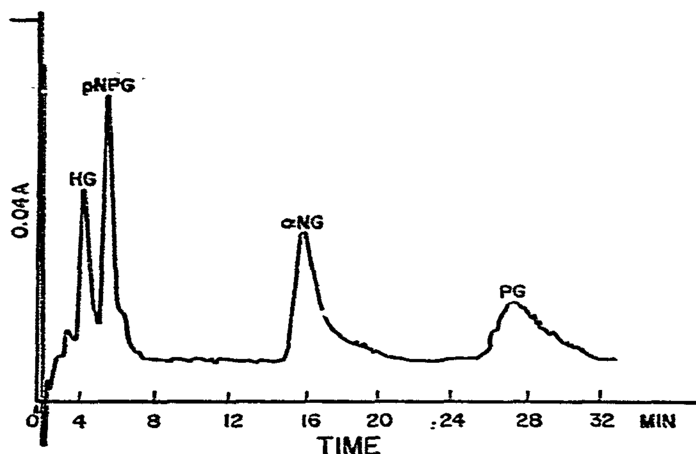

Fig. 1. Chromatography of 0.02% solutions of harmol glucuronide (HG), *p*-nitrophenyl glucuronide (pNPG), α-naphthyl glucuronide (αNG) and phenolphthalein glucuronide (PG). Eluent: 0.01 *M* tetrabutylammonium bromide in 35% methanol in water. Flow-rate, 0.8 ml/min and chart speed, 30 cm/h.

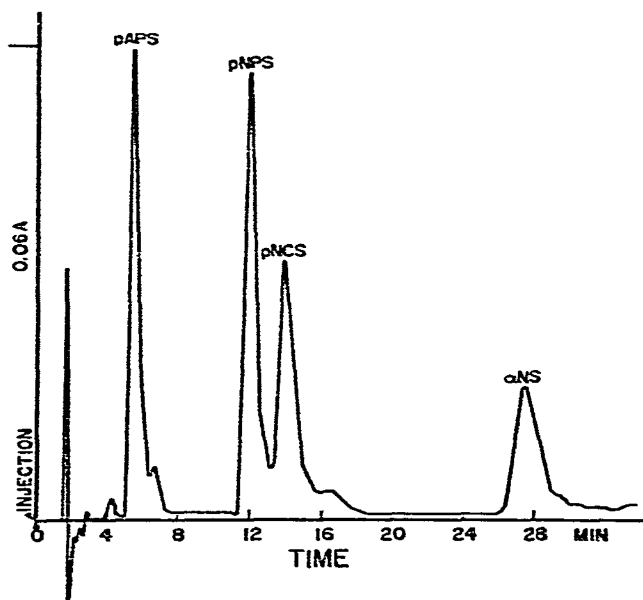

Fig. 2. Chromatography of 0.02% solutions of *p*-acetylphenyl sulfate (pAPS), *p*-nitrophenyl sulfate (pNPS), *p*-nitrocatechol sulfate (pNCS) and α-naphthyl sulfate (αNS). Eluent: 0.01 *M* tetrabutylammonium bromide in 40% methanol in water. Flow-rate, 0.8 ml/min and chart speed, 30 cm/min.

Ion-pair reversed-phase HPLC has been shown to separate readily model systems of a molecule and its glucuronide and sulfate conjugates and mixtures of different glucuronides and different sulfates. The system may be useful in estimating the concentration of conjugates in biological fluids in metabolite studies and clinical situations after appropriate separation from endogenous materials.

ACKNOWLEDGEMENTS

One of the authors (A. K.) was supported by a faculty fellowship from the University of Ankara, College of Pharmacy, Ankara, Turkey.

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