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Note

Separation of phenols and their glucuronide and sulfate conjugates by anion-exchange liquid chromatography

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Improved methods are needed for both separation and detection of polar, foreign compound metabolites in biological tissues and fluids. The most important mammalian conjugation reactions involve attachment of glucuronide and sulfate moieties, with glucuronidation predominating[1,2]. Accordingly, our method development efforts have been focused on these two conjugate classes.

Gas chromatography (GC) and GC–mass spectrometry (MS) have been used to measure glucuronides, but isolation and derivatization are necessary[3]. Sulfate conjugates are generally hydrolized by acid or enzymatically, followed by analysis of the non-polar hydrolysis product[3]. The use of high-performance liquid chromatography (HPLC) offers the potential for the direct determination of sulfate and glucuronide conjugates with little or no sample preparation.

Various HPLC methods for these compounds have been published, most of these based on ion-pair reversed-phase chromatographic systems[4–10]. In each of these reports, the authors separated either a single phenol and its glucuronide and sulfate, or the sulfate or glucuronide conjugates of several different phenols. In contrast, the present paper describes the simultaneous separation of three phenols and their respective glucuronide and sulfate conjugates using a strong anion-exchange chromatographic column and an ammonium formate buffer, an application that has not previously been reported.

EXPERIMENTAL

Chemicals

Phenyl-β-D-glucuronide, 1-naphthyl-β-D-glucuronide, 4-nitrophenyl-β-D-glucuronide, and 1-naphthyl sulfate potassium salt were purchased from Sigma (St. Louis, MO, U.S.A.). Phenyl sulfate potassium salt was purchased from TCI American (Portland, OR, U.S.A.). 4-Nitrophenyl sulfate was prepared in our laboratory as potassium salts using a modification of the procedure of Burkhardt and Lapworth[11] as described elsewhere[12]. The commercial and synthetic compounds were confirmed by negative fast atom bombardment MS and high-field proton nuclear magnetic resonance spectroscopy[12].

Standards were prepared in either distilled, deionized water or pesticide grade methanol (EM Science, Cherry Hill, NJ, U.S.A.). The mobile phase consisted of

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pesticide grade acetonitrile (EM Science) and aqueous ammonium formate buffer. The ammonium formate buffer was prepared by adjusting the pH of a 0.05 *M* formic acid solution to 4.5 with concentrated ammonia (J. T. Baker, Phillipsburg, NJ, U.S.A.). The formic acid was 96% and was obtained from Merck (Rahway, NJ, U.S.A.).

C.A. registry numbers. Phenol: [108-95-2]; phenyl sulfate potassium salt: [1733-88-6]; phenyl glucuronide: [17685-05-1]; 4-nitrophenol: [100-02-7]; 4-nitrophenyl sulfate potassium salt: [6217-68-1]; 4-nitrophenyl glucuronide: [10344-94-2]; 1-naphthol: [90-15-3]; 1-naphthyl sulfate potassium salt: [6295-74-5]; 1-naphthyl glucuronide: [17238-47-0].

HPLC system

The chromatographic system consisted of an Isco Model 2300 HPLC pump and an Isco V^4 variable-wavelength absorbance detector (Lincoln, NE, U.S.A.). A Valco C6W injection valve (Houston, TX, U.S.A.) with a 10-μl sample loop was used for sample introduction. The chromatograms were recorded on a Spectra-Physics (San Jose, CA, U.S.A.) Model 4290 digital integrator. The separations were carried out on a Supelco (Bellefonte, PA, U.S.A.) 25 cm × 4.6 mm 5-μm LC-SAX column.

Experimental procedure

A mixed standard solution containing nine target compounds, each at approximately 100 ng/μl was used. The compounds were phenol, phenyl glucuronide, phenyl sulfate potassium salt, 4-nitrophenol, 4-nitrophenyl glucuronide, 4-nitrophenyl sulfate potassium salt, 1-naphthol, 1-naphthyl glucuronide and 1-naphthyl sulfate potassium salt. Serial dilutions were made of the stock solution to give solutions of approximately 50, 25, 12, and 6 ng/μl of each component. The mixtures were chromatographed isocratically with a mobile phase of buffer–acetonitrile (3:2, v/v) at a total flow-rate of 1.5 ml/min. Compounds were detected by absorbance at 254 nm and identified by retention time. Calibration curves were constructed by plotting peak area *vs.* concentration.

TABLE I

CAPACITY FACTORS AND EFFICIENCY OF LC-SAX COLUMN

Compound	*Retention time (min)*	*Capacity factor*	*Number of theoretical plates*
Void volume	2.36	–	–
Phenol	2.78	0.18	4300
4-Nitrophenol	3.14	0.33	5500
1-Naphthol	3.48	0.48	6700
Phenyl glucuronide	5.37	1.28	7100
4-Nitrophenyl glucuronide	5.79	1.45	8300
1-Naphthyl glucuronide	7.15	2.03	5600
Phenyl sulfate	11.0	3.68	17 000
4-Nitrophenyl sulfate	13.4	4.68	16 100
1-Naphthyl sulfate	19.5	7.26	12 000

RESULTS AND DISCUSSION

The retention times, capacity factors and numbers of theoretical plates for each compound are shown in Table I, and a typical chromatogram is shown in Fig. 1. Complete separation is achieved within 25 min. The calibration curves for the nine compounds are all linear, with correlation coefficients of 0.996 or greater. Limits of detection based on peak heights three times the peak-to-peak baseline noise range from 9 ng for phenol to 270 ng for phenyl sulfate.

The LC–UV procedure described may be readily adapted to HPLC–MS. A commonly used interface for HPLC–MS is the thermospray (TSP) interface, which uses a volatile buffer, generally ammonium acetate or ammonium formate, to effect ionization[13]. The mobile phase used with the SAX column would therefore be compatible with HPLC–TSP–MS. Particle beam interfaces do not require an ionizing buffer, and could therefore use ion pairing reagents[13]. However, such reagents contaminate the interface and the mass spectrometer source[14]. This SAX method is therefore also well suited to particle beam LC–MS. While ammonium formate was used in this work, we found that ammonium acetate gives very similar results[14].

While the LC-SAX column gave very efficient and reproducible separations, it was very sensitive to storage conditions. Column performance was maintained by rinsing the column at the end of the day with about 45–60 ml of a phosphate solution, prepared by adding concentrated phosphoric acid to a 0.05 *M* solution of K_2HPO_4 to

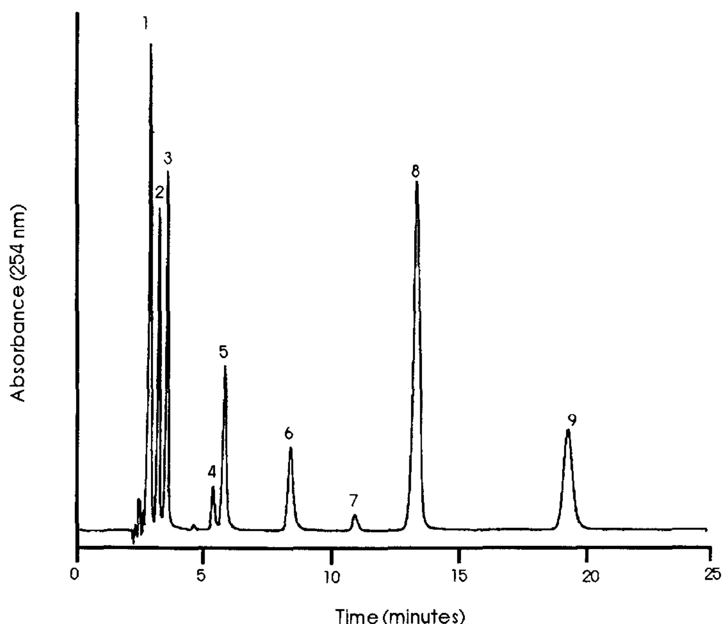

Fig. 1. Sample chromatogram of 100 ng/μl standard. Peaks: 1 = phenol; 2 = 4-nitrophenol; 3 = 1-naphthol; 4 = phenyl glucuronide; 5 = 4-nitrophenyl glucuronide; 6 = 1-naphthyl glucuronide; 7 = phenyl sulfate potassium salt; 8 = 4-nitrophenyl sulfate potassium salt; 9 = 1-naphthyl sulfate potassium salt.

a pH of 2.5. Failure to store the column in the phosphate solution would result in a severe decrease in retention time, especially for the sulfates. The original chromatographic characteristics of the column could be restored by storage in the phosphate solution for one to two days.

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