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High-speed liquid chromatography of glucuronide and sulfate conjugates

High-speed liquid chromatography is a relatively new form of liquid chromatography in which the column is operated at high pressures (> 1000 p.s.i.g.) permitting rapid analyses to be made. Systems capable of operation under these conditions have been described by several authors[1-5]. These systems have been employed in biochemical studies for the analysis of nucleotides and related compounds[1,4,6-8], carbohydrates[9], amino acids[10] and urinary constituents[11,12]. Recently high-speed liquid chromatography has been applied to the analysis of various drugs including benzodiazepines[13], phenacetin metabolites[14] and barbiturates, diphenylhydantoin and their hydroxylated metabolites[15].

Few methods are available for the qualitative and quantitative analysis of drug conjugates in biological samples. For example, thin-layer chromatography has been employed for the analysis of the glucuronide and sulfate conjugates of *p*-hydroxyacetanilide[16] and gas chromatography has been utilized in glucuronide analysis[17-19]. No methods have been described for the direct gas chromatographic analysis of sulfate conjugates although derivatives of these compounds can be analyzed by this technique[20]. The success obtained with the use of high-speed liquid chromatography in the analysis of various compounds of biochemical interest suggested that this technique could be useful for the analysis of sulfate and glucuronide conjugates. The present paper describes conditions for the analysis of sulfate and glucuronide conjugates in urine using high-speed liquid chromatography; a preliminary report of this work has been presented elsewhere[21].

Experimental

Chemicals. Phenol, *p*-nitrophenol, catechol, *p*-hydroxyacetanilide, phenyl glucuronide, *p*-nitrophenyl glucuronide, *p*-nitrophenylsulfate, formic acid and potassium chloride were used as obtained from the suppliers. Modifications of the methods of DUSZA *et al.*[22] and HEARSE *et al.*[23] were employed for the synthesis of N-acetyl-*p*-aminophenyl sulfate (acetanilide sulfate) and catechol monosulfate. N-Acetyl-*p*-aminophenyl glucuronide (acetanilide glucuronide) was isolated by thin-layer chromatography (*n*-butanol–acetic acid–water (8:1:1); acetone–*n*-butanol–water (5:4:1)[16] from the urine of rats given 150 mg/kg *p*-hydroxyacetanilide intraperitoneally or from the urine of adult human males given 15 mg/kg *p*-hydroxyacetanilide (Tylenol®) orally. Spots corresponding to acetanilide glucuronide on thin-layer chromatograms showed a positive reaction to the naphtharesorcinol test for glucuronides. Similarly, peaks corresponding to acetanilide glucuronide collected from the liquid chromatograph were glucuronide positive using the method of MEAD *et al.*[24]. Acetanilide glucuronide and sulfate peaks showed the presence of *p*-aminophenol after collection and analysis[25,26].

Apparatus. A Varian LCS-1000 high-speed liquid chromatograph equipped with an ultraviolet photometer monitoring absorbance at 254 mμ was employed. The stainless-steel column (1 mm I.D. × 250 cm long) contained a pellicular anion-exchange resin[2] (type LSF); the column was maintained at 80°. Inlet pressures in the range of 800–1000 p.s.i.g. provided a flow rate of 30 ml/h. Exact instrumental operating parameters are given in the tables and figures.

Procedure. Experiments were conducted employing both non-gradient and gradient modes of operation. When conditions were varied, the system was flushed and the column purged with the new solution for at least 30 min. Formic acid, 10.0 m*M*, pH 3 containing 1.0 *M* potassium chloride served as the eluent when non-gradient elution was employed. The following conditions were employed in most gradient elution experiments: high concentrate solution: 1.0 m*M* formic acid, pH 4, containing 2.0 *M* potassium chloride; gradient chamber solution: 1.0 m*M* formic acid, pH 4; initial volume of 1.0 m*M* formic acid in gradient chamber: 50 ml; high concentrate flow rate into the gradient chamber: 15 ml/h and column flow rate: 30 ml/h. These conditions yield a linear gradient of potassium chloride.

Sample preparation. Reference materials were dissolved in double distilled water for analysis. Collected urine was immediately passed through an ultrafiltration membrane (Centriflo®, Amicon Corp.) and the ultrafiltrates stored frozen until analyzed by direct injection into the liquid chromatograph.

Results and discussion

The non-gradient mode of operation was selected for initial experiments dealing with the high-speed liquid chromatography of phenols and their glucuronide and sulfate conjugates. Trials with eluents of differing molarity and pH were performed. Formate, acetate, and phosphate buffers of pH greater than 5.0 and varying in molarity from 1.0 m*M* to 1.0 *M* failed to adequately resolve phenols and their corresponding glucuronides; in addition poor peak shape was observed. These buffers did not elute sulfate conjugates. Addition of potassium chloride to the above buffers did not significantly improve resolution. Failure of the previously mentioned buffers to elute sulfate conjugates led to the use of eluents of lower pH and increased ionic strength. Formic acid solutions, pH 3.0, containing 1.0 *M* potassium chloride successfully eluted sulfate conjugates as well as phenols and glucuronides (Fig. 1 and Table I).

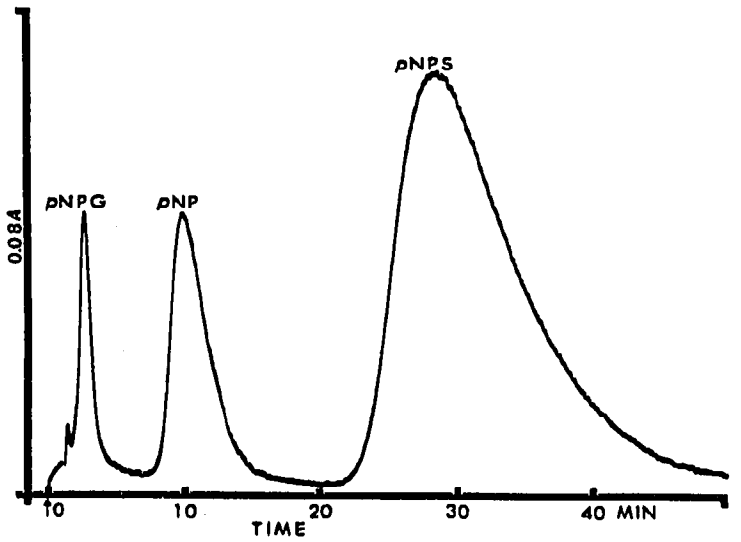

Fig. 1. Chromatography of *p*-nitrophenol (*p*NP, 100 nmoles), *p*-nitrophenyl glucuronide (*p*NPG, 50 nmoles) and *p*-nitrophenyl sulfate (*p*NPS, 150 nmoles). Mode: non-gradient; eluent: 10.0 m*M* formic acid, pH 3, containing 1.0 *M* potassium chloride; column temperature: 80°; flow rate: 30 ml/h; inlet pressures: 800–1000 p.s.i.g.

TABLE I

RETENTION VALUES OF PHENOLS AND THEIR CORRESPONDING GLUCURONIDE AND SULFATE CONJUGATES

Compound	*Non-gradient mode, retention time (min)*[a]	*Gradient mode, retention time (min)*[b]
Phenol	5.0	5.0
Catechol	4.7	4.7
p-Nitrophenol	15.5	13.9
p-Hydroxyacetanilide	3.6	3.4
Phenyl glucuronide	3.1	9.7
Catechol glucuronide	2.7	3.1
p-Nitrophenyl glucuronide	4.5	3.7
Acetanilide glucuronide	2.7	8.5
Phenyl sulfate	12.5	—[c]
Catechol monosulfate	14.7	—[c]
p-Nitrophenyl sulfate	38.5	—[c]
Acetanilide sulfate	9.5	37.5

[a] Conditions: 10.0 m*M* formic acid, pH 3, containing 1.0 *M* potassium chloride, 80°, 30 ml/h.

[b] Conditions: Low concentrate chamber: 1.0 m*M* formic acid, pH 4. High concentrate chamber: 1.0 m*M* formic acid, pH 4, containing 2.0 *M* potassium chloride. Flow of high concentrate into gradient chamber: 15 ml/h. Column flow: 30 ml/h. Initial volume: 50 ml. Temperature: 80°.

[c] No compound eluted within 90 min.

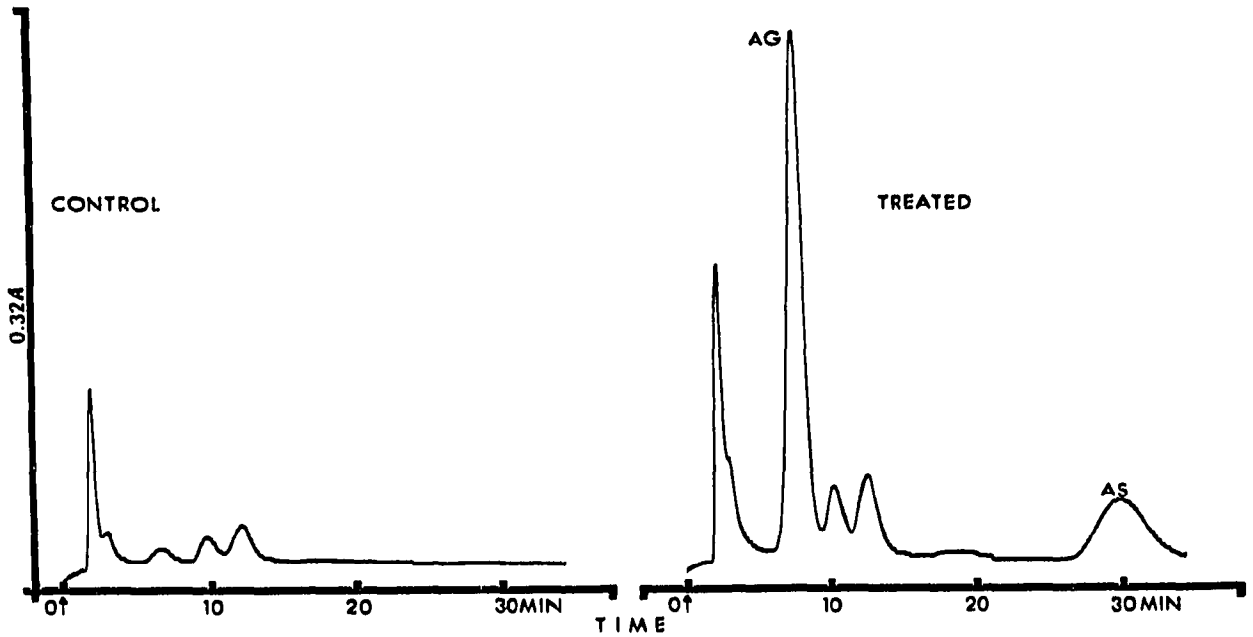

Fig. 2. Chromatography of *p*-hydroxyacetanilide metabolites in human urine. Subject was normal adult human male given 15 mg/kg *p*-hydroxyacetanilide (Tylenol®) orally. One microliter of ultrafiltrate from control and 0–5 h urine samples was injected into the liquid chromatograph. AG = acetanilide glucuronide, AS = acetanilide sulfate. Mode: gradient; eluent: 1.0 m*M* formic acid, pH 4 to 1.0 m*M* formic acid, pH 4, containing 2.0 *M* potassium chloride; temperature: 80°; flow rate: 30 ml/h; inlet pressures: 800–1000 p.s.i.g.

It should be noted, though, that the non-gradient system did not satisfactorily retain glucuronide conjugates on the column.

Experiments using gradient elution techniques were undertaken in an attempt to provide satisfactory analytical conditions for glucuronides. A linear gradient formed by mixing 1.0 m*M* formic acid, pH 4.0, with 2.0 *M* potassium chloride in 1.0 m*M* formic acid, pH 4, separated phenols and glucuronides (Table I). Gradient elution also resolved a mixture of *p*-hydroxyacetanilide, acetanilide glucuronide and acetanilide sulfate. This gradient system did not elute phenyl, *p*-nitrophenyl or catechol sulfate. The data in Table I shows that relatively little difference in retention times of phenols was observed between non-gradient and gradient modes of operation; similar results were obtained with *p*-nitrophenyl or catechol glucuronide. In contrast to the results obtained with non-gradient operation, the gradient system retained phenyl and acetanilide glucuronides longer than the corresponding phenols.

The methods described above have been applied to the estimation of conjugates in biological fluids. Specifically, non-gradient elution techniques have been used to measure sulfate conjugates in chicken urine following the infusion of catechol and *p*-nitrophenol as well as phenyl sulfate itself[27]. Gradient elution permitted analysis of phenyl glucuronide in chicken urine following its infusion. In other experiments acetanilide glucuronide and sulfate have been identified and quantitated in both rat and human urine following the administration of *p*-hydroxyacetanilide (Fig. 2). Urine samples were prepared and analyzed as described in the *Experimental* section. Previously BURTIS *et al.*[14] have reported the liquid chromatography of phenacetin metabolites. They observed the presence of glucuronide conjugates of *p*-hydroxyacetanilide and 3-methoxy-4-hydroxyacetanilide. However, sulfate conjugates were not detected and the elution time for acetanilide glucuronide was approximately 23 h. In contrast, the method reported in this paper permits quantitative estimation of acetanilide glucuronide and sulfate in urine in less than 40 min. These results suggest that high-speed liquid chromatography should be useful for monitoring glucuronide and sulfate conjugates of drugs in clinical situations.

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A sensitive method for the detection of adenine compounds separated by paper or thin-layer chromatography

Purine compounds separated by paper chromatography (PC) or thin-layer chromatography (TLC) are most often detected by means of their fluorescence in UV light[1]. Purines can also be detected by PC by directing UV light at chromatograms pinned over a sheet of photographic paper; upon development of the photographic paper, the UV absorbing purines appear as white spots against a dark background[2]. Location reagents are less sensitive than the above techniques for locating purines[1]. One location reagent is the silver nitrate–Bromphenol Blue reagent of WOOD[3]. This reagent forms a blue purine-silver-dye complex which, under optimum conditions, can detect 0.05 μmole of purine derivatives.

In the present communication paper chromatograms are first dipped in an acidic vanillin reagent; they are then dried and dipped in a modified silver nitrate–Bromphenol Blue reagent. Adenyl compounds appear as pink spots on a gold background. The method is considerably more sensitive than any of the methods described above and specifically detects adenyl purines only. The same method can be applied to TLC by spraying instead of dipping. The specificity and sensitivity of the method are discussed.

Materials and methods

All the materials and reagents used were commercially available. The chromatographic paper used was Whatman No. 1. Microcrystalline cellulose, vanillin and silver nitrate were obtained from Merck AG, Darmstadt, G.F.R. Bromphenol Blue was obtained from May & Baker Ltd., Dagenham, Great Britain.