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Short communication

Porous graphitic carbon shows promise for the rapid chromatographic analysis of polar drug metabolites

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Abstract

A rapid and simple HPLC method has been developed and used to separate the polar metabolic conjugates of AZT, chloramphenicol, and β-estradiol based upon the use of porous graphitic carbon. The HPLC system is sufficiently selective to resolve the polar drug conjugates from their parent compounds and from endogenous material present in urine. The compounds are separated, without the need for sample pretreatment or gradient elution, on a porous graphitic carbon (Hypercarb) column using aqueous trifluoroacetic acid modified with tetrahydrofuran as the mobile phase. Porous graphitic carbon exhibits a novel mechanism of retention towards these very polar substances, which are unretained under reversed-phase conditions on alkyl-bonded silica phases.

1. Introduction

Drug metabolism is a complex process. This complexity is illustrated by the wide range of metabolic reactions of drugs reported in the literature [1,2]. In general, metabolism introduces polar functionality into drug molecules; this increases aqueous solubility of the metabolite relative to the parent drug and so facilitates excretion in urine. Typical examples of this process are conjugation with carbohydrates, such as glucuronic acid, or with sulphate or amino acids [3–5].

The efforts of mammalian systems in rendering drug molecules more polar in order to effect excretion also provides the analyst with a challenge on occasions where there is a need to quantify or monitor the polar metabolite in a biological matrix such as urine.

Urine contains many polar endogenous components and achieving resolution of polar analytes from urinary components is a particularly difficult task. Gradient elution has been used to overcome the problems caused by analytes with widely different polarities in complex matrices [6–9]. Other approaches include chemical or enzymatic hydrolysis of the conjugates with analysis of the products [10] and ion-exchange chromatography [11,12]. All of these techniques can suffer disadvantages such as long analysis time or complicated sample preparation.

Avoiding these problems in biological fluids would require a very different selectivity from that of conventional reversed-phase HPLC. The alternative selectivity offered by porous graphitic carbon (PGC) stationary phase, developed by

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Knox et al. [13], showed promise in that it has been used for the analysis of compounds with a wide range of polarities, including the very polar creatinine and oxalic acid [14]. In this paper we describe the application of PGC to the analysis of polar metabolites in biological fluids, using three drug glucuronides in urine as model systems. The method is shown to be rapid and selective.

2. Experimental

2.1. Reagents

Trifluoroacetic acid, 3'-azido-2'-deoxycytidine (AZT), AZT glucuronide, chloramphenicol glucuronide and 17-β-estradiol glucuronide were obtained from Sigma (Poole, Dorset, UK). Methanol (HPLC grade) was obtained from Rathburn Chemicals (Walkburn, Scotland, UK). Tetrahydrofuran (HPLC grade) and perchloric acid were purchased from BDH (Poole, Dorset, UK). Triethylamine (sequanal grade) was obtained from Pierce (IL, USA), fluparoxan (GR50360) and ^{3}H-radiolabelled fluparoxan were synthesised at Glaxo Research and Development (Greenford, Middlesex, UK). Fluparoxan glucuronide and sulphamate metabolites were isolated from dog urine after dosage with radiolabelled fluparoxan and characterised by mass spectroscopy and NMR.

2.2. Apparatus

The analyses were performed using a Model HP1090A liquid chromatograph equipped with a diode-array detector and an autosampler (Hewlett-Packard, Palo Alto, CA, USA). Radiochemical isotope detection was performed by means of a Berthold radiochemical detector equipped with a liquid scintillation cell, (Berthold Instruments, Berlin, Germany). Chromatographic separations were carried out on a 100 × 4.6 mm I.D., 7 μm Hypercarb column, supplied by Shandon Scientific (Runcorn, Cheshire, UK).

2.3. Mobile phases

The mobile phases were prepared by computer-controlled electronic mixing of methanol, tetrahydrofuran, aqueous trifluoroacetic acid, concentration 1.0%, and water. An eluent flow-rate of 1.0 ml min^{-1} was used throughout.

2.4. Test solutions

Each glucuronide conjugate was dissolved in water separately to produce a 1 mg ml^{-1} solution. AZT was dissolved in water to produce a solution containing 1 mg ml^{-1}. Fluparoxan was dissolved in methanol to produce a solution of 1 mg ml^{-1}. Fluparoxan glucuronide and sulphamate were isolated from dog urine by semi-preparative HPLC. The compound structures are shown in Fig. 1.

2.5. Ultraviolet detection

Initial experiments revealed that the model conjugates could be detected by ultraviolet spectrophotometry using the optimum wavelengths of 270, 280 and 280 nm, respectively for AZT glucuronide, chloramphenicol glucuronide and β-estradiol glucuronide, or 280 nm as a compromise fixed wavelength. The glucuronides and parent compounds had similar UV spectra.

2.6. Selection of chromatographic conditions

Solutions of the three model glucuronide conjugates, AZT glucuronide, chloramphenicol glucuronide and 17-β-estradiol glucuronide, were individually injected onto the PGC column. Initially the chromatography was carried out using a mainly aqueous eluent modified with either methanol, acetonitrile, 2-propanol, dioxan or tetrahydrofuran. None of the conjugates were eluted by any of the organic modifiers, even with a 90% modifier concentration.

Guanghua and Lim [14] have described the analysis of creatinine and oxalic acid in urine using PGC with a highly aqueous mobile phase containing 1% trifluoroacetic acid. Using this methodology the three model conjugate solu-

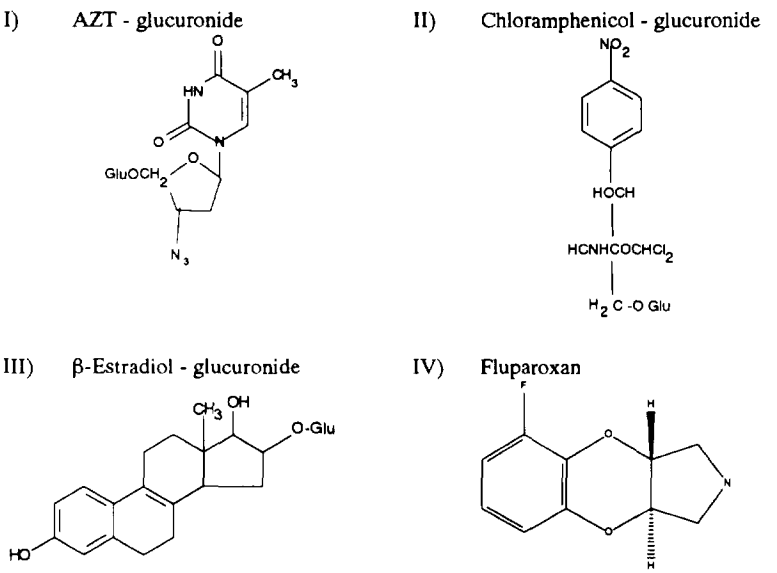

Fig. 1. Structures of the conjugate compounds. I = AZT glucuronide; II = chloramphenicol glucuronide; III = 17-β-estradiol glucuronide; IV = fluparoxan; Glu = glucuronide conjugate.

tions were injected onto the PGC column, no elution was observed for any of the conjugate compounds. Each of the organic solvents previously employed were then, in turn, individually added to the acid/aqueous mobile phase. Only the AZT glucuronide was eluted with methanol as the organic modifier and this required a very high concentration (85%, v/v); however, all of the compounds were successfully eluted from the column when tetrahydrofuran was employed as the organic modifier at a relatively low concentration (40%, v/v). Compound integrity was confirmed by diode-array comparison of the UV spectra of the eluting peaks. All further work was carried out with acidic/aqueous mobile phase using tetrahydrofuran as the organic modifier.

3. Results and discussion

The percentage tetrahydrofuran content of the mobile phase can be used to control the retention of the glucuronide conjugates as shown in Fig. 2. The retention time of each of the three glucuronide conjugates was determined at several different tetrahydrofuran concentrations from 0 to 90% (v/v), by three replicate injections of each analyte with each mobile phase composition. In each case, the retention time of the glucuronide conjugate decreased as the concentration of the modifier was increased. The plots of logarithm of capacity ratio, k', versus modifier concentration show an approximately linear relationship, with a negative gradient. As can be

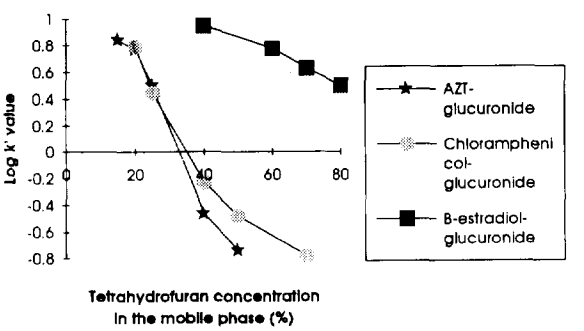

Fig. 2. Plot of log k' for glucuronide conjugates of AZT, chloramphenicol and 17-β-estradiol versus organic modifier concentration.

seen the scatter of the experimental data is far more marked than that normally found with alkyl-bonded silicas and organic polymeric phases. This, when coupled with the strong retention of these polar compounds on PGC, suggests the existence of a different retention mechanism in the case of porous graphitic carbon to that exhibited by alkyl-bonded silicas and polymeric phases.

Whereas the retentions of the glucuronides gradually decreased with increasing modifier concentration the effect of the acid strength upon elution was quite different. The pH of the mobile phase was gradually increased by increments of 0.5 of a pH unit, from pH 1 to pH 7, and the retention time of each of the conjugates was determined for each pH value. In the range from pH 1 to ≈ 5 the hydrogen ion concentration of the mobile phase appeared to have a little or no effect on compound retention, however, at pH values greater than 5 the drug conjugates were not eluted. It is likely that the abrupt change observed is due to changes in the state of ionization of glucuronide conjugate molecules as the pH of the mobile phase changes. At low pH amine groups on the AZT and chloramphenicol molecule will be ionized and so be attracted to the aqueous mobile phase. As the pH increases these groups will become progressively less ionised and so the compound will be retained longer on the column. As the pH is increased above 7 then the hydroxyl groups on the glucuronide molecule will be deprotonated making the compound once again attracted to the mobile phase and thus compound retention will be reduced.

Using porous graphitic carbon and tetrahydrofuran–aqueous trifluoroacetic acid eluents the conjugates are readily separated from their parent compounds without the need for gradient elution, as illustrated by the chromatogram in Fig. 3. In all three examples the parent drug molecule eluted after the glucuronide conjugate. The stability of the glucuronide conjugates on the PGC surface was evaluated by stop-flow chromatography. No chemical degradation of any of the glucuronides was observed on the PGC stationary phase. However, when this experiment was repeated using a C_6 bonded-silica phase a small but significant amount of parent compound was observed from the AZT-glucuronide.

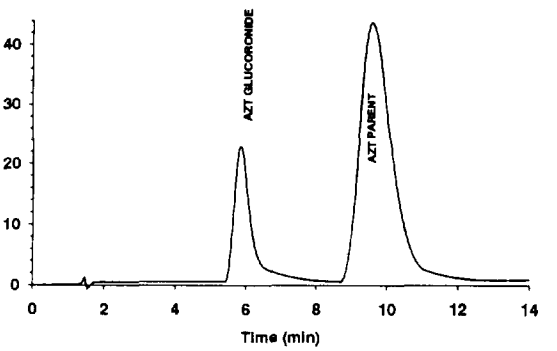

Fig. 3. Separation of AZT and AZT glucuronide. Chromatographic conditions: column, Hypercarb (7 μm, 100 × 4.6 mm I.D.); mobile phase, tetrahydrofuran–water–trifluoroacetic acid (30:69.5:0.5, v/v); temperature, 25°C; flow-rate, 1.0 ml min^{-1}; injection volume, 10 μl; detection, UV at 280 nm.

The mobile phase was easily adjusted to resolve the glucuronide conjugates from the endogenous material present in urine when directly injected onto the HPLC system (Fig. 4) (the separation of AZT glucuronide and endogenous material in urine). In order to completely resolve the AZT glucuronide from endogenous material in urine it was necessary to reduce the concentration of tetrahydrofuran in the mobile phase from 30% (v/v) to 25% (v/v). As the organic solvent concentration had a more profound effect upon the retention time of the analyte molecule than on the endogenous material in urine, lowering the modifier concentration increased the analyte retention and thus produced compound resolution. The presence of the AZT glucuronide was confirmed by its UV spectrum and examination of a urine blank.

In addition to model conjugates the HPLC system has been applied successfully to the separation of the parent and metabolites of the novel alpha-2-adrenoceptor antagonist fluparoxan, which after oral administration to the dog forms a carbamyl glucuronide and sulphamate conjugate. Again the use of porous graphitic carbon gave rise to satisfactory separations for

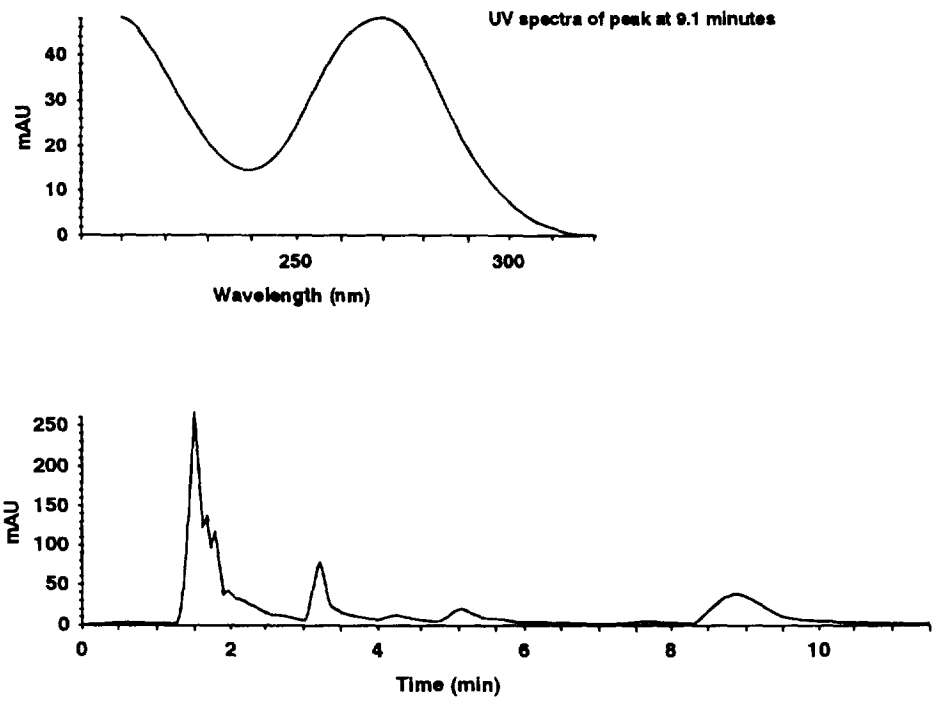

Fig. 4. Separation of AZT glucuronide from endogenous material in urine. Chromatographic conditions: column, Hypercarb (7 μm, 100 × 4.6 mm I.D.); mobile phase, tetrahydrofuran–water–trifluoroacetic acid (25:74.5:0.5, v/v); temperature, 25°C; flow-rate, 1.0 ml min^{-1}; injection volume, 10 μl; detection, UV at 280 nm.

the parent compound and its conjugates, as illustrated by the chromatograms in Fig. 5. The resolution of the two metabolites could only be achieved with an organic modifier concentration of 30% (v/v); at this concentration the parent molecule fluparoxan was unretained. However, at lower concentrations of organic modifier the fluparoxan molecule was retained. The metabo-

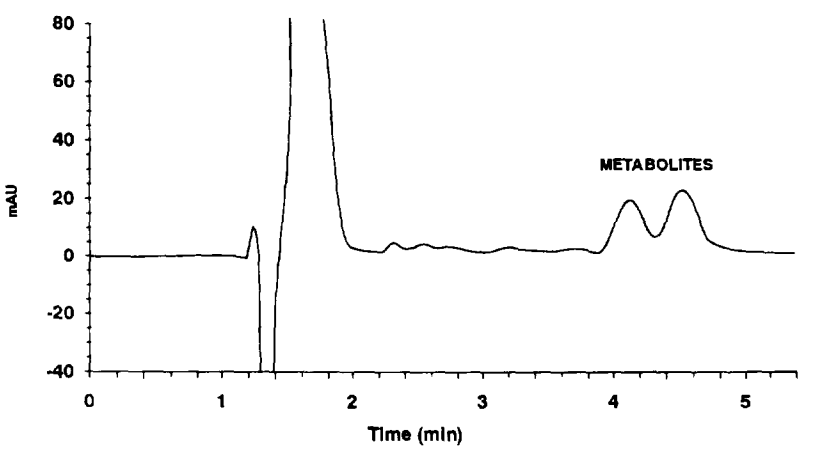

Fig. 5. Separation of fluparoxan from its metabolites. Chromatographic conditions: column, Hypercarb (7 μm, 100 × 4.6 mm I.D.); mobile phase, tetrahydrofuran–water–trifluoroacetic acid (30:69.5:0.5, v/v); temperature, 25°C; flow-rate, 1.0 ml min^{-1}; injection volume, 10 μl; detection, UV at 280 nm.

lites were isolated from in vitro metabolic studies in the dog, using radiolabelled fluparoxan. These metabolites were identified as being related to fluparoxan by the use of a HPLC radiochemical detector. As no authentic standard existed for the sulphamate or glucuronide conjugate the order of metabolite elution has not been elucidated.

Unlike the model conjugates, in this case the parent compound eluted before the metabolites. As the metabolites were both unretained in reversed-phase chromatography, even with trace amounts of organic modifier, this suggests that the order of compound elution is not solely determined by the polarity of the molecule. Closer examination of this fact may yield useful information on the mechanism of retention which has yet to be explained satisfactorily.

When examined by reversed-phase HPLC on octadecylsilica phases the model conjugates were found to be weakly retained, and eluted with the solvent front even when only small quantities of organic modifier were used. In contrast, on porous graphitic carbon the glucuronides were strongly retained and resolved from their respective parent compounds. The material has proved sufficiently selective, with minor adjustments to the mobile phase, to separate the polar metabolites from their parent molecules and also from the endogenous material present in urine.

4. Conclusions

The results clearly demonstrate that porous graphitic carbon (PGC) can be used to analyse polar glucuronide conjugates without the need for gradient elution or chemical/enzymatic hydrolysis of the conjugates. The PGC columns generally have efficiencies lower than that of conventional silica-based high-performance columns, at around 20–30 000 plates/m, but the novel selectivity and ability to retain highly polar compounds compensates for the lower efficiency when analysing polar compounds. As PGC is based on a two-dimensional form of graphite its mechanism of retention is different to that of silica-bonded phases and has yet to be explained satisfactorily and will be the subject of further studies in the authors' laboratories.

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