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NMR-monitored solid-phase extraction of phenolphthalein glucuronide on phenylboronic acid and C_{18} bonded phases*

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Abstract: Preliminary studies have been undertaken to evaluate the potential of immobilized phenylboronic acid (PBA) for the solid-phase extraction (SPE) of glucuronide metabolites from urine. These studies have demonstrated that immobilized PBA can be used to specifically extract phenolphthalein glucuronide (5 mM) from urine. Urine samples were loaded onto the PBA SPE column in glycine buffer (pH 8.5) and were eluted using methanol–1% HCl (90:10, v/v). The overall recoveries of the phenolphthalein glucuronide for this procedure were high (99%), which compared well with similar studies carried out concomitantly on C_{18} bonded columns (93%).

Keywords: *^{1}H-NMR; solid-phase extraction; phenylboronic acid; glucuronides.*

Introduction

High resolution proton nuclear magnetic resonance (NMR) spectroscopy has proved to be extremely useful for the direct detection, identification and quantification of drugs, drug metabolites and endogenous components in biofluids [1]. This is a result of the ability of NMR to provide rapid multicomponent metabolite determination of a complex biological sample with the minimum of physical or chemical pre-treatment. Methods have been developed that utilize solid-phase extraction and NMR spectroscopy (SPE–NMR) for the isolation and identification of drug metabolites in a biofluid [2–4]. To date, the bulk of this work has employed C_{18} bonded silica gel with ion suppression to retain ionizable metabolites, or ion-exchange materials.

Prior to excretion, many foreign compounds undergo extensive metabolism. Such biotransformations can have important consequences for the therapeutic action or toxicity associated with the compound(s) under investigation. Thus, there is a clear requirement for their metabolic fate to be determined. One of the main metabolic reactions that occurs is conjugation of glucuronic acid to xenobiotics. Glucuronic acid can be conjugated to a wide variety of chemical groups, e.g. hydroxyl, carboxylic, amino and sulphydryl groups, to form the corresponding glucuronides. In the past, C_{18} bonded SPE columns have been used to isolate and purify glucuronides [2–4]. Preliminary investigations on the use of a potentially more specific phase, immobilized phenylboronic acid (PBA), are reported. The retention of suitable compounds on PBA (e.g. catecholamines, carbohydrates, nucleosides and vicinal diols) [5–8] is facilitated by both covalent and non-specific mechanisms. The energetics of the covalent bond are high when compared to those of reversed-phase mechanisms. Advantages of covalent bonding result from the fact that while the compound of interest remains bound to the column, com-

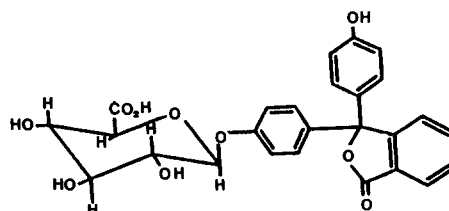

Figure 1
Structure of phenolphthalein glucuronic acid (PHGA).

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pounds retained by non-specific mechanisms can be eluted. The application of SPE–NMR using PBA to extract phenolphthalein glucuronide (Fig. 1) from urine is reported. The results for PBA are compared to those obtained on the C_{18} bonded silica gel column.

Experimental

NMR Spectroscopy

^{1}H-NMR spectra were recorded on a JEOL GSX 500 spectrometer operating at 11.75 Tesla field strength (500 MHz ^{1}H frequency) at ambient probe temperature (25°C). Sixty-four free induction decays (FIDs) were collected for each sample into 32,768 computer points using a spectral width of 6000 Hz and a data acquisition time of 2.73 s. A further delay of 2.27 s between pulses was used to ensure that the spectra were fully T_1 relaxed. The intense signal from the residual water protons was suppressed by applying a secondary gated irradiation field at the water resonance frequency. An exponential line broadening factor of 0.2 Hz was applied prior to Fourier transformation. Chemical shifts were referenced to sodium 3-trimethyl silyl-[2,2,3,3,$^2H_4$]-1-propionate (TSP; $\delta = 0$ ppm).

Sample preparation

Control urine from a normal healthy subject was collected in a polypropylene tube and stored frozen at −30°C until use. Sufficient test sample, comprised of urine spiked with phenolphthalein glucuronic acid (Sigma, Poole, UK), was prepared such that the final concentration was 5 nM. An aliquot (500 µl) of this sample was freeze dried and reconstituted in 600 µl of ^2H_2O, as an internal frequency-field lock, and placed into a 5 mm (o.d.) glass NMR tube.

Solid-phase extraction onto C_{18} SPE columns

Five hundred microlitres of the spiked, acidified urine samples (pH = 2, using 2 M HCl) was loaded onto a C_{18} Bond-Elut^{TM} column (Varian Assoc., CA, USA, obtained from Jones Chromatography Ltd, Hengoed, Mid. Glamorgan, UK), containing 500 mg of sorbent, that had been activated by washing with 5 ml of methanol followed by 5 ml of acidified water (pH = 2). Acidification ensured the retention of the phenolphthalein glucuronide on the column. In order to obtain selective recovery of the glucuronide, step-wise elution procedures were employed. The retained compounds were eluted with 1 ml of methanol–acid water mixtures of increasing elutropic strength, i.e. 20:80, 40:60, 60:40, 80:20 and 100% methanol, into scintillation vials. After removal of the methanol using a stream of nitrogen followed by freeze drying to remove the residual water, each fraction was reconstituted in 600 µl ^2H_2O containing TSP as an internal reference.

Solid-phase extraction with PBA columns

Five hundred microlitres of a urine sample with 500 µl of 100 mM glycine buffer (pH = 8.5) were loaded onto the PBA column (Varian Assoc., obtained from Jones Chromatography Ltd). The columns, which contained from 100 to 400 mg of PBA, were equilibrated prior to sample application with 5 ml of 0.1 M glycine buffer (pH = 10) followed by 5 ml of glycine buffer (pH 8.5) in order to obtain the reactive boronate form $RB(OH)_3^-$. Phenolphthalein glucuronide was then eluted with 5 ml of methanol–1% HCl (90:10, v/v). The methanol was removed and each fraction was freeze-dried to remove residual water, with a final reconstitution in 600 µl of ^2H_2O containing TSP as an internal reference.

Results and Discussion

The 500 MHz ^{1}H-NMR spectrum of freeze-dried control urine that had been reconstituted in ^2H_2O is shown in Fig. 2(A). The spectrum reveals several frequency ‘windows’ (e.g. 0–1, 1.5–1.9, 5–7 and 7.8–10 ppm) that contain few resonances from endogenous compounds, thus allowing signals due to phenolphthalein glucuronide (Fig. 2B) to be observed without difficulty (Fig. 2C). Acidification, solid-phase extraction of 500 µl of the phenolphthalein glucuronide-containing urine sample on a C_{18} column, with subsequent step-wise gradient elution using methanol-acidified water mixtures of increasing elutropic strength resulted in the recovery of the phenolphthalein glucuronide in three fractions (Fig. 3). Initial elution of the urine sample followed by an acidified water wash and then 20% methanol (Fig. 3B) led to the removal of endogenous compounds such as creatinine, citrate, and hippurate. Elution with 40% methanol resulted in the recovery of 54% of the glucuronide while subsequent elution with 60% methanol re-

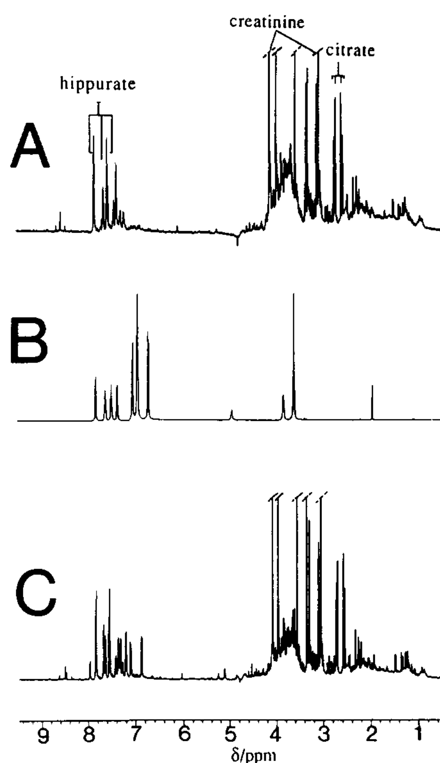

Figure 2
500 MHz ^{1}H-NMR spectra of (A) control human urine, (B) phenolphthalein glucuronide and (C) control human urine spiked with phenolphthalein glucuronide (5 mM).

sulted in the recovery of a further 34% of the glucuronide (Fig. 3C and D). Small quantities were also recovered in the 80% methanol extract. Clearly, having removed the contaminating co-extracted endogenous substances with the 20% methanol-acidified water wash, elution and recovery of the phenolphthalein glucuronide could have been accomplished with a single 80% methanol wash. The total of phenolphthalein glucuronide recovered in all three fractions was about 93%.

The extraction of phenolphthalein glucuronide by the immobilized PBA was then studied. A proposed mechanism of covalent bond formation to the PBA is illustrated in Fig. 4 [8]. The column was first equilibrated with an alkaline solution to obtain the reactive boronate form $RB(OH)_3^-$. The analyte was then applied and covalently bound with the concomitant release of water. Once the analyte was retained, contaminants could be selectively washed from the bonded phase, provided an alkaline pH was maintained. Finally, the compound of interest was eluted by acidification of the eluting solvent, thus releasing it from the boronate complex. The covalent bonding to the PBA is a function of the pH

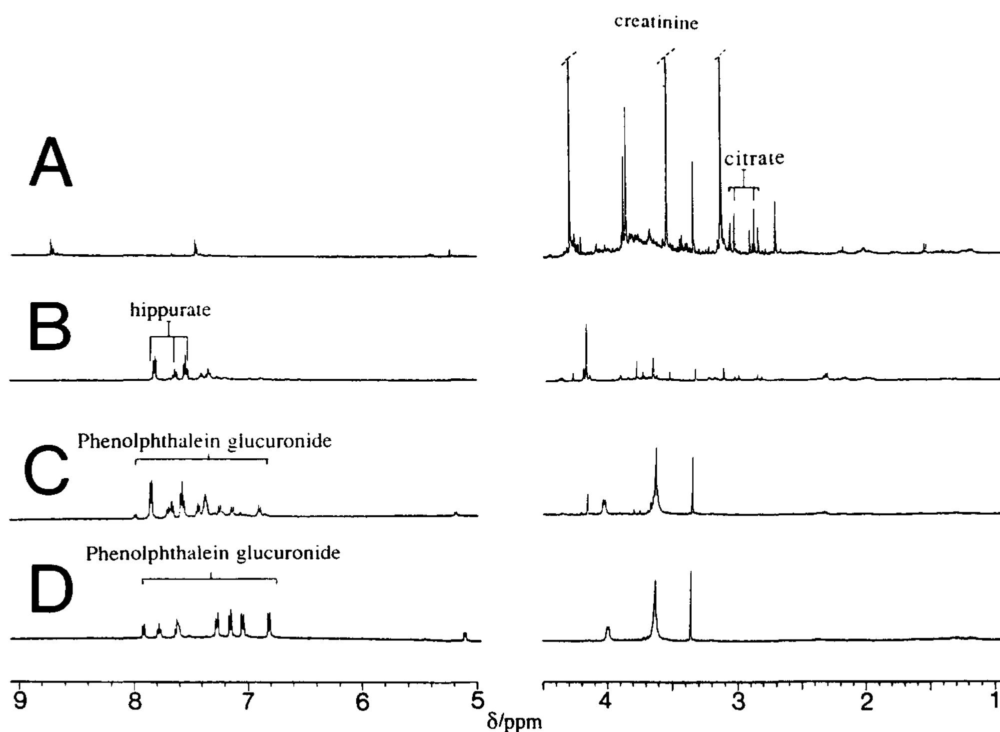

Figure 3
500 MHz ^{1}H-NMR spectra of control human urine containing phenolphthalein glucuronide (PHGA) after solid-phase extraction on a C_{18} Bond Elut^{TM} column. (A) C_{18} non-retained material, (B) 20% methanol eluate, (C) 40% methanol eluate and (D) 60% methanol eluate.

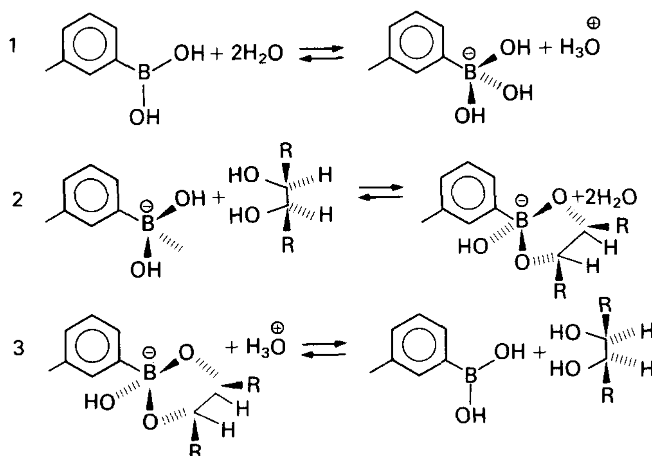

Figure 4
Phenylboronic acid (PBS): the proposed three step process of boronate binding including equilibration, retention and elution [8].

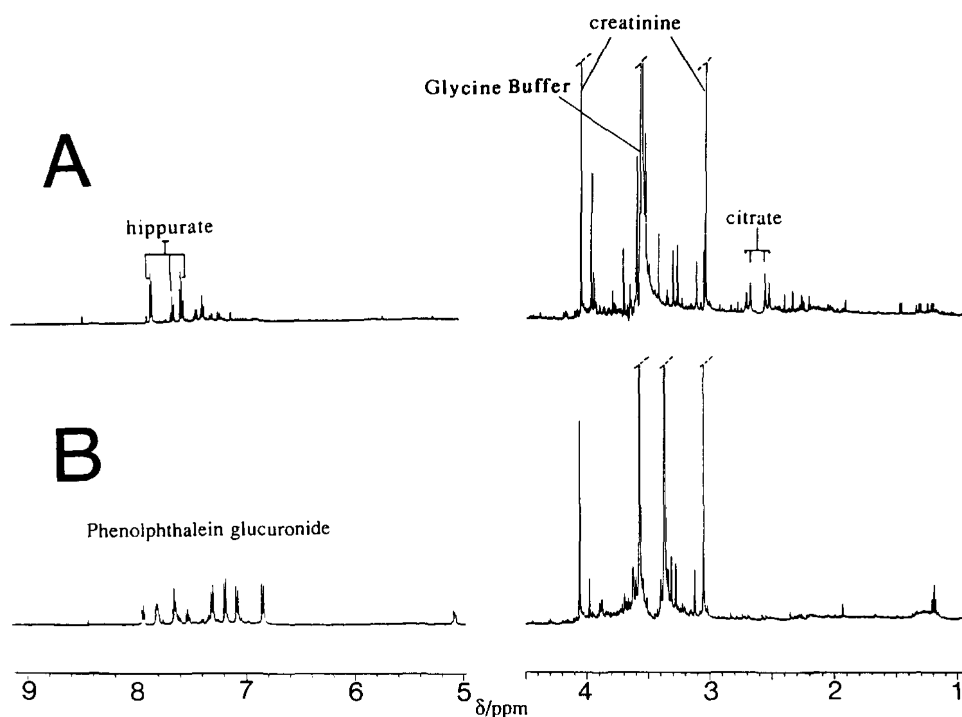

Figure 5
500 MHz ^{1}H-NMR spectra of control human urine containing phenolphthalein glucuronide after solid-phase extraction on a PBA column. (A) Non-retained material, (B) methanol–1% HCl (90:10, v/v) eluate.

(the pK_a of the immobilized phenylboronic acid is approximately 9.2) and it was essential to properly equilibrate the bonded phase with an alkaline solution to obtain good extraction efficiency for the phenolphthalein glucuronide. In the initial experiments with PBA, the phenolphthalein glucuronide was not completely retained on the column (100 mg of PBA) and some of the analyte was lost during the initial elution used to remove endogenous substances. Therefore, further experiments were performed to determine the amount of PBA that had the required capacity to retain all of the glucuronide. Four PBA columns containing different amounts of material were prepared. The elution profiles obtained revealed that 300 mg of PBA retained the bulk of the phenolphthalein glucuronide. All of the

phenolphthalein glucuronide was extracted onto a column that contained 400 mg of PBA. The results obtained for extraction onto a 400 mg PBA column are shown in Fig. 5(A). The initial elution profile of the sample resulted in the elimination of essentially all of the endogenous urinary metabolites (e.g. citrate, creatinine, hippurate and alanine) (Fig. 5A). The retained phenolphthalein glucuronide was recovered (96%) in a single step by elution with methanol–acidified water (90:10, v/v) essentially free of endogenous substances (apart from a small quantity of creatinine) (Fig. 5B).

The mechanism of covalent bond formation between PBA and the analyte, shown in Fig. 4, assumes that the glucuronyl moiety is being bound as a 1,2-diol. Clearly, however, the possibility that the glucuronide is bound via the β-hydroxy carboxylic acid cannot be discounted without first undertaking appropriate studies with esterified glucuronides. In addition, other secondary interactions (e.g. hydrophobic π–π interactions with the phenyl ring of the PBA) may also contribute to the binding of the phenolphthalein glucuronide to the PBA column.

Irrespective of the exact mechanism, the results of these preliminary experiments have revealed the selectivity and extraction potential of the PBA column for glucuronides and have illustrated the differences between the PBA and C_{18} phases. Should the ability of PBA to extract glucuronides from biological fluids prove to be a general property of this stationary phase, then it may provide a simple, rapid and specific procedure for the isolation of this important class of drug metabolites.

Further studies directed at fully defining the properties of these columns for the extraction of glucuronides and to determine whether PBA can be used to concentrate trace glucuronides from larger volumes of urine are in progress.

References

[1] J.K. Nicholson and I.D. Wilson, *Prog. NMR Spectros.* **21**, 449–501 (1989).

[2] I.D. Wilson and J.K. Nicholson, *Anal. Chem.* **59**, 2830–2832 (1987).

[3] I.D. Wilson and J.K. Nicholson, *J. Pharm. Biomed. Anal.* **6**, 151–165 (1987).

[4] I.D. Wilson and I.K. Ismail, *J. Pharm. Biomed. Anal.* **4**, 663–665 (1986).

[5] R.R. Maestas, J.R. Priesto, G.D. Kuehn and J.H. Hageman, *J. Chromatogr.* **189**, 225–231 (1980).

[6] F.H. Pfadenhauer and S.D. Tong, *J. Chromatogr.* **162**, 585–590 (1979).

[7] A.H.B. Wu and T.G. Garnet, *Clin. Chem.* **31**, 298–302 (1985).

[8] M.L. Stolwitz, in *Proceedings of the Second Annual International Symposium on Sample Preparation*, pp. 41–44. Analytichem International, CA, USA (1985).

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