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IMMOBILIZED BORONIC ACIDS: EXTRACTION

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Introduction

With the exceptions of antibody and molecular imprint-based methods, most solid-phase extractions rely on relatively nonspecific nonpolar van der Waals or ionic interactions. Another exception to the use of nonspecific interactions involves the use of reversible covalent bond formation with vicinal diols, or similar structures, in the target analyte with immobilized boronic acids. Clearly, the potential to exploit this type of interaction is limited but, where it can be exploited, highly specific solid-phase extraction (SPE) methods can result. Such methods, based on the use of boronic acids immobilized on materials, such as sepharose gels or phenylboronic acid (PBA) covalently linked to silica gel, have provided the basis for a number of SPE methods, as described below.

Mechanism of Interaction of Analytes with Immobilized PBA

The extraction mechanism that results in the formation of cyclic boronates is illustrated in **Figure 1**. In order for the reaction to proceed, the boronate must be in the reactive $-B(OH)_3^-$ form, which is readily obtained by equilibrating the phase with an alkaline buffer. When the sample is applied, the covalent bond forms only with analytes possessing suitable functional groups, e.g. vicinal diols, found in sugars or catechols. Other functional groups which can form covalent bonds with boronic acids include α-hydroxy acids, aromatic *O*-hydroxyacids and amides, 1,3-dihydroxy-, diketo-, triketo- and aminoalcohol-containing compounds. With formation of the covalent bond, the analyte is strongly bound to the phase, which may then be washed with strongly eluotropic solvents to remove nonspecifically retained contaminants (an alkaline pH must be maintained). Analytes can then be recovered using an acidic buffer/solvent, which hydrolyses the covalent bonds to liberate retained compounds and return the PBA to the $-B(OH)_2$ form.

As well as these specific interactions with PBA, a number of nonspecific interactions can also occur with residual silanols, the aminopropyl group via which the PBA is attached to the silica, and the phenyl ring itself, which offers the opportunity for π–π interactions. In addition, the boronic acid can act as a hydrogen bond donor, cations can also bind to the boronic acid, and there is the potential for the formation of charge-transfer complexes with unprotonated amines. All of these interactions may happen when performing an extraction, and care must be taken to ensure that the extraction scheme is optimized to the required boronate retention mechanism if the maximum specificity is to be obtained.

Buffer Selection

The first criterion to ensure a good extraction efficiency is to select a buffer for extraction on to

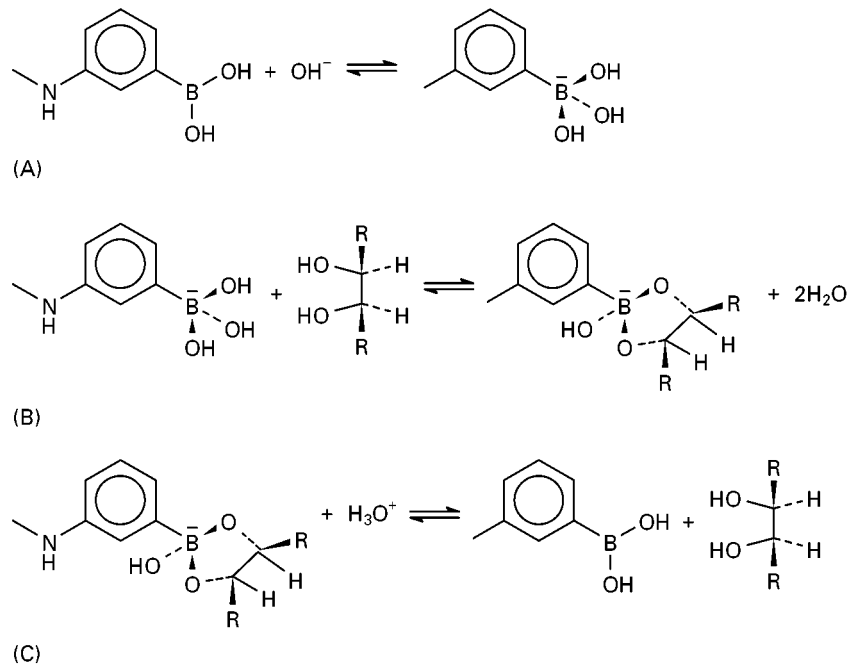

Figure 1 Mechanism of boronic acid extraction. (A) Activation of boronic acid in the presence of alkali; (B) formation of a covalent bond with a vicinal diol; (C) the use of acid to break the covalent bond and regenerate the boronic acid and the free diol.

PBA with an alkaline pH for the conditioning and sample application steps. Equilibration can be performed with, e.g. 0.1–1.5 mol L^{-1} buffer at pH 10–12 and then 0.01–0.05 mol L^{-1} buffer at pH 8–8.5. Zwiterionic buffers such as HEPES, glycine, diglycine and morpholine offer advantages and all have been used in this type of application. Clearly, buffers which can form covalent adducts with PBA are to be avoided, for the obvious reason that they eliminate covalent bond formation with the analyte. Such buffers include bicine, tricine, tris and 1′,2′,3′-ethanolamine.

Having obtained retention, analytes can be recovered by reducing the pH of the eluent to 5 or below. Typically acetic, trifluoroacetic and phosphoric acids may be used, with the addition of an organic modifier to help overcome nonpolar or silanophilic secondary interactions. Occasionally, the covalent adduct is sufficiently stable to require the addition of lactic or salicylic acid to the eluting buffer. Alternatively, elution with borate-containing buffers can be employed when acid-labile analytes are present.

Applications

Endogenous Biochemicals

Adenosine, catecholamines, dopamine, DOPA and related substances A major field of application in the use of SPE with immobilized boronates (both gels and silica-based materials) is the extraction of various endogenous substances, especially catecholamines, l-dihydroxyphenylalanine (DOPA) and related materials, from biofluids. However, here we have concentrated on descriptions of the more recent procedures, generally involving the use of silica-based materials.

PBA gels have been employed in the extraction of nucleosides from biological samples for several studies, including the isolation of inosine and adenosine from human plasma. Methods have also been described for the simultaneous extraction of the adenosine and dopamine from human urine using a silica-based PBA phase. In this case extraction was performed using 100 mg PBA, activated by washing first with 5 mL of 0.1 mol L^{-1} formic acid followed by 5 mL pH 8.8 ammonium acetate buffer (0.25 mol L^{-1}). Urine (0.5 mL, pH 8.8) containing (±)-isoproterenol and 2-chloroadenosine, was allowed to flow through the sorbent bed until the liquid meniscus just reached the top of the layer, at which point 1 mL of pH 8.8 ammonium acetate (0.25 mol L^{-1}) was also applied to the cartridge. Elution was achieved with 1 mL of 0.1 mol L^{-1} HCl–methanol (4 : 1 v/v). This methodology allowed recoveries of 88–104% to be attained with good coefficients of variation (less than 5%).

An interesting two-stage SPE method has been devised for the isolation of DOPA from plasma and

urine using [^{14}C]-DOPA as an internal standard. Interfering urinary pigments (urochromes) were eliminated via an initial extraction on to a dual-layer cartridge consisting of an upper layer of strong cation exchanger (SCX) and a lower layer of Cl silica. After this cartridge had been conditioned with 5 mL methanol and HCl (0.2 mol L^{-1}) the urine sample (2.8 mL) was applied followed by a further two bed volumes of 0.2 mol L^{-1} HCl. The eluate was taken to pH 7.5–7.7 (1.5 mL of 2 mol L^{-1} Tris buffer), after which an aliquot was passed through 200 mg of PBA SPE column (conditioned with 1 mL of methanol and 1 mL of 0.2 mol L^{-1} Tris buffer). After removing of interferences with methanol (2 mL) and 0.1 mol L^{-1} Tris (1 mL), the analyte was eluted in 0.3 mL of 0.1 mol L^{-1} HCl. For plasma samples, it was necessary to remove plasma proteins prior to extraction. This was done via precipitation using ice-cold perchloric acid. The supernatant obtained after centrifugation was then passed through the PBA cartridge (at pH 7.5–7.7 with 2 mol L^{-1} Tris) and the analyte subsequently eluted, as described above. Overall recoveries of 80% for urine and 84% for plasma (SD 2–3%) were obtained (allowing 10–15 pg to be detected with high performance liquid chromatography (HPLC) electrochemical detection).

As well as DOPA itself, several methods have used PBA for extracting 5-*S*-*L*-cysteinyl-*L*-dopa (5-SCD) from urine. These methods have included a dual extraction, first on to a cation exchanger and then a PBA gel with 5-*S*-*D*-diastereoisomer as an internal standard. A more recent method using PBA on silica was also based on dual extraction with SCX and PBA. The urine samples, to which 5-*S*-*D*-cysteinyl-*L*-dopa (D–CD) had been added as an internal standard, were applied to an SCX column that had been washed sequentially with methanol (1.0 mL) and HCl (0.1 mol L^{-1}, 1 mL). After washing with HCl (0.1 mol L^{-1}) the SCX cartridges were placed in series with a PBA column (pretreated with methanol and then 1 mol L^{-1} dipotassium phosphate buffer). The target compounds were eluted from the SCX using the dipotassium hydrogen phosphate buffer. Compounds retained on the PBA cartridge were then eluted with 0.5 mL of 0.1 mol L^{-1} HCl (containing 10 mg L^{-1} of ascorbic acid), following a water wash step. Aliquots of the eluate were then analysed by HPLC.

More recently a new, fully automated method for 5-*S*-cysteinyldopa in plasma and urine has been proposed. In this method, the PBA cartridges were treated sequentially with heptane and acetone (1 mL of each) to remove impurities and were then conditioned with methanol (1 mL), HCl (0.3 mol L^{-1}, 1 mL) and Tris buffer (10 mmol L^{-1}, 2 mL) followed by application of the appropriately buffered sample (1 mL of plasma or 1 : 100 diluted urine). The cartridges were then washed with 4 mL of 10 mmol L^{-1} Tris buffer prior to elution of the analyte with HCl (1 mL of 0.3 mol L^{-1}). Typical chromatograms from this work are illustrated in **Figure 2**.

Boric acid gels and silica–PBA have both been used to extract catecholamines from biological samples. The silica-based material has also been employed for the determination of noradrenaline and adrenaline in urine and found to be superior to conventional methods. In this method two SPE cartridges, a pentanesulfonic acid phase (PSA) and PBA were connected in series (the PBA cartridge had previously been washed with methanol (1 mL) and then HCl (0.1 mol L^{-1}, l mL)). Both cartridges were then washed sequentially with methanol (2 mL) and then aqueous ammonia (4 mL) and finally phosphate buffer (4 mL, 5 mmol L^{-1} pH 8.5). The urine sample (1 mL, pH 5 using ammonia) plus internal standard (dihydroxybenzylamine) were then applied followed by a wash with phosphate buffer (4 mL of pH 8.5 phosphate). After washing with a further 2 mL of the alkaline phosphate buffer, the PSA column was removed and the PBA cartridge was washed first with methanol (1 mL) and then acetonitrile–phosphate buffer (1 mL, 1 : 1 v/v). Recovery of the analytes was achieved using HCl (1 mL, 0.1 mol L^{-1}) and these were then analysed by HPLC with electrochemical detection.

In a similar method, urine (1 mL, diluted to 5 mL with water, pH 6.5–7.0) being assayed for dopamine, adrenaline and noradrenaline with 3,4-dihydroxybenzylamine added as internal standard was extracted using a combination of SCX and PBA. The SCX and PBA cartridges were initially treated with 1 mol L^{-1} HCl followed by methanol and 0.01 mol L^{-1} ammonium acetate buffer (pH 7.3), following which the sample was applied to the SCX column. This was washed with methanol and then ammonium acetate (0.01 mol L^{-1}): recovery of the analytes from this phase was achieved with 3×500 μL of perchloric acid. This eluate was neutralized using a saturated solution of sodium carbonate and was then loaded on to the PBA cartridge which was first washed with methanol and then water. Finally the analyte was recovered for analysis, by HPLC with electrochemical detection, by elution with 2×500 μL of 0.1 mol L^{-1} perchloric acid. Limits of detection of 1 μg L^{-1} for noradrenaline and 2 μg L^{-1} for dopamine were claimed.

Glycosylated amino acids In diabetes the glycosylated amino acid glucitollysine is formed when the

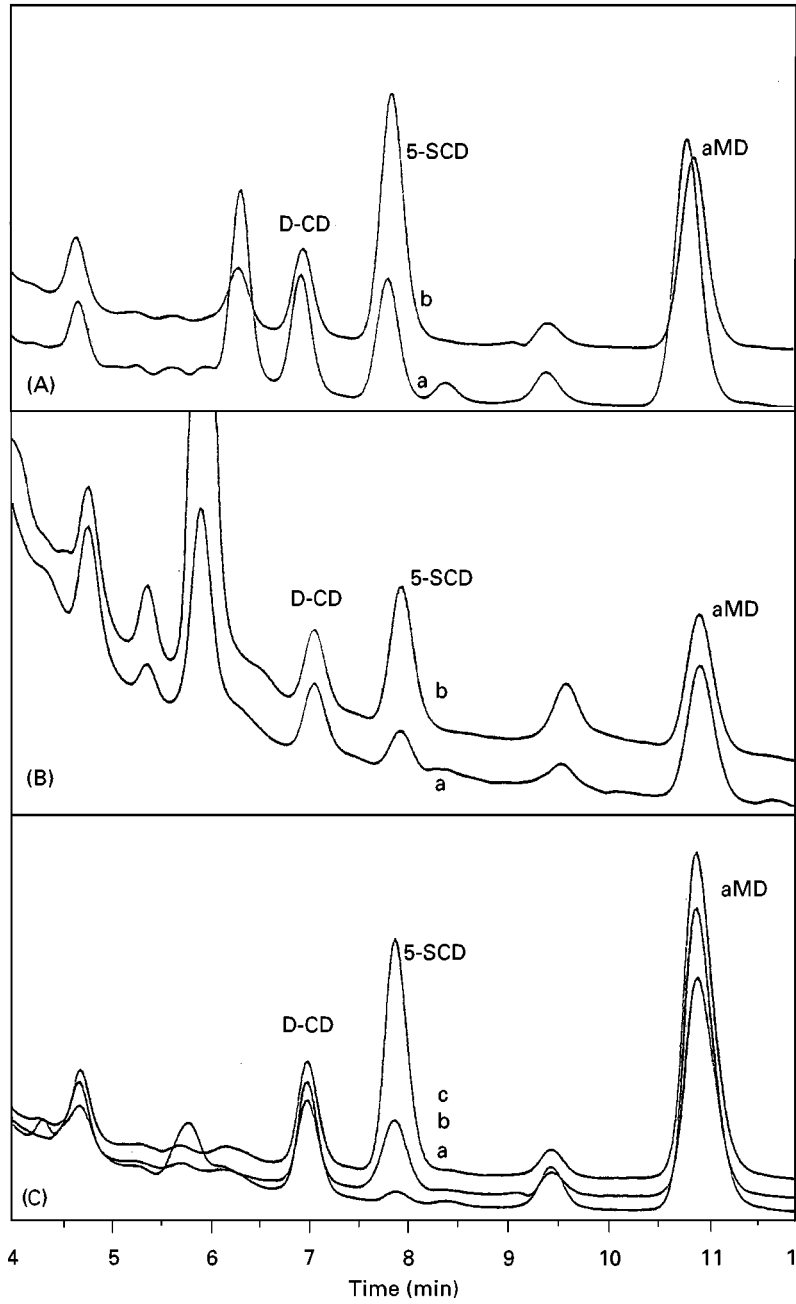

Figure 2 Chromatograms of 5-*S*-cysteinyldopa in PBA-extracted aqueous calibration standards plasma and urine. (A) Urine samples: (a) normal 5-SCD concentration (320 μg L^{-1}) and (b) pathological 5-SCD concentration (1310 μg L^{-1}). (B) Plasma samples: (a) nomal 5-SCD concentration (1.4 μg L^{-1}) and (b) pathological 5-SCD concentration (5.3 μg L^{-1}). (C) Extracted aqueous calibration standards: 5-SCD concentration: (a) 0.4 μg L^{-1}; (b) (3.2 μg L^{-1}; (c) 8.0 μg L^{-1}. aMD, 2-methyl-3-C3,4-dihydroxyphenyl)-L-alanine Reproduced with permission from Hartleb *et al.* (1999).

amino acid lysine in proteins reacts with glucose. An online extraction method for glucitollysine in protein hydrolysates with 'on-column' reaction with *o*-phthaldialdehyde (OPA) to allow HPLC with fluorescence detection has been described.

Glucitollysine extraction was achieved by washing the PBA phase with 0.1 mol L^{-1} HCl to remove contaminants, followed by 0.1 mol L^{-1} NaOH, equilibration with pH 8.5 phosphate buffer (0.1 mol L^{-1}) and then application of the sample, also at pH 8.5. With the protein hydrolysates, interfering co-extracted amino acids were removed by washing with water or methanol. Recovery of glucitollysine was achieved by lowering the pH of the mobile phase.

Reduced oligosaccharides PBA has also been applied to the separation of oligosaccharides from their alditols and interfering amino acids and glycopeptides. The purification of an oligosaccharide–lipid conjugate (neoglycolipid) formed by the reductive amination of the sugar lactose with phosphatidylethanolamine dipalmitoyl (PPEADP) has also been demonstrated.

The columns were activated by treatment with methanol, HCl (0.1 mol L^{-1}), water and then NaOH (0.2 mol L^{-1}). Following washing with water (2×1 mL) samples were applied as aqueous solutions. Elution was performed by washing with water, acetic acid (0.1 mol L^{-1}) and finally HCl (0.1 mol L^{-1}) with the fractions eluted from the PBA analysed by thin-layer chromatography on silica. Under these conditions, oligosaccharides with glucose at the reducing end were not retained by the PBA columns, but the corresponding alditols were retained. Similar results were obtained for glycoprotein-derived octasaccharides and their corresponding alditols. The application of samples under alkaline conditions enabled the separation of the analytes from amino acids, peptides and glycopeptides, although there was some retention of nonreducing oligosaccharides.

In addition, methods were also provided that enabled the purification of oligosaccharide derivatives formed by reductive amination (resulting in the ring-opened sugars giving acylic vicinal hydroxyl groups). Column activation in this example was performed using water, methanol and a 1 : 1 (v/v) mixture of methanol–chloroform to wash the column. Reaction mixtures obtained after reductive amination were applied in methanol–chloroform (1 : 1 v/v), with subsequent elution (after various washes) in chloroform–methanol–0.1 mol L^{-1} acetic acid (30 : 70 : 30 v/v).

Natural Products

Polyhydroxyflavones HPLC with sample preparation via extraction on to PBA cartridges has recently been applied to the analysis of a variety of dietary polyhydroxyflavones (quercetin, kaempferol, fisetin, rutin, myricetin and morin; see **Figure 3** for structures) present in vegetables, red wine and human blood plasma. The extraction involved conditioning the cartridges with aqueous acetonitrile (1 mL, 28% v/v) containing 1% trifluoroacetic acid followed by 1 mL water and 1 mL of phosphate buffer (0.5 mol L^{-1}, pH 8.5). The samples were then loaded on to the cartridge in phosphate buffer (pH 8.5). Following a wash step (1 mL of 10 mmol L^{-1} phosphate buffer, pH 8.5) the analytes were recovered in 2 mL of the aqueous acetonitrile solution used in the first step of the cartridge conditioning process (applied in four 0.5 mL aliquots). In general, good recoveries of the target compounds were obtained from matrices such as red wine and onions; recovery of, for example, quercitin was always greater than 90%. From human plasma the recovery of this compound was reduced to *c.* 80%, but with quite acceptable inter- and intraassay coefficients of variation. In **Figure 4** chromatograms for a variety of sample types are illustrated following sample preparation on PBA.

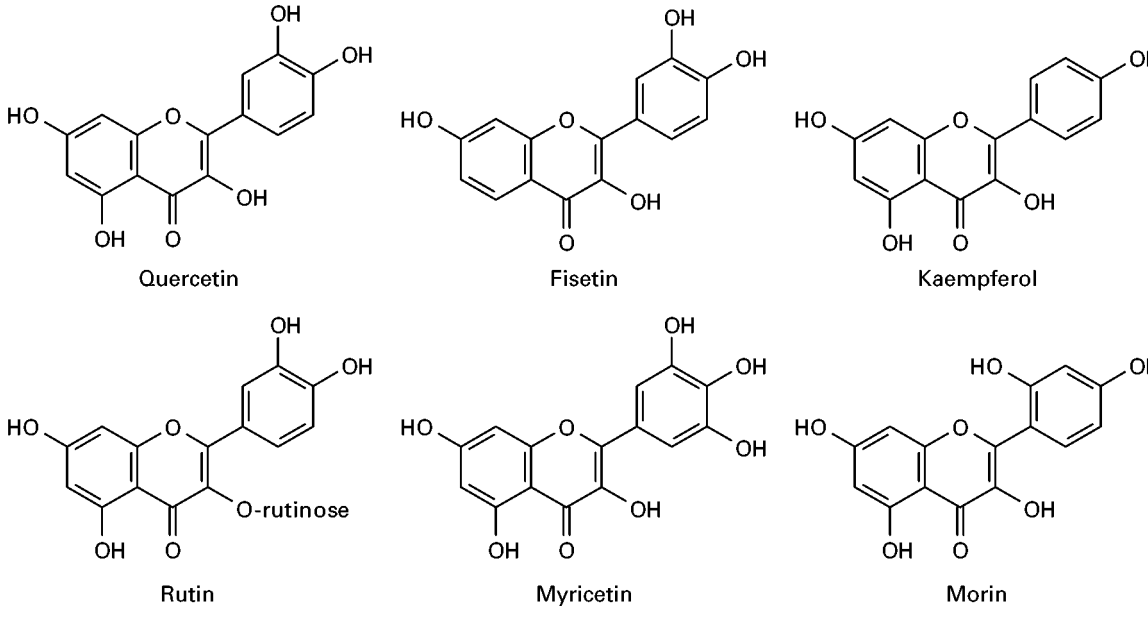

Figure 3 Structures of the dietary polyhydroxyflavones.

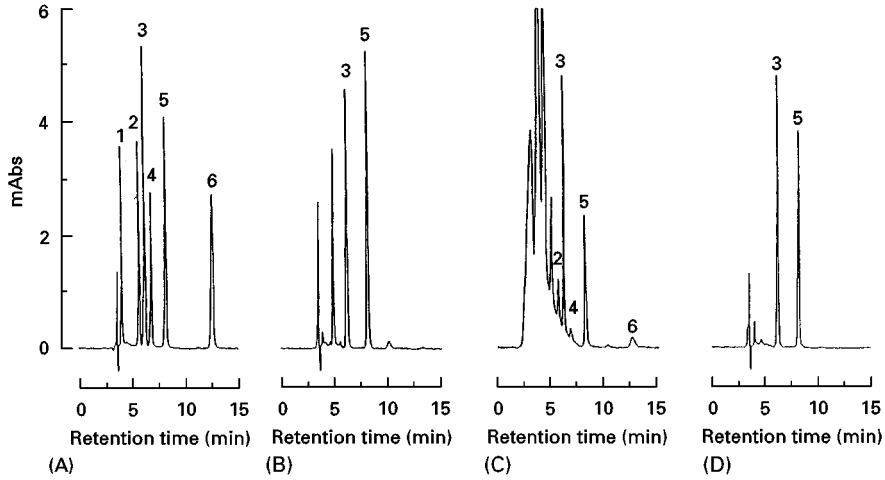

Figure 4 Chromatograms for a variety of sample types containing dietary polyhydroxyflavones following sample preparation on PBA. (A) Standard polyhydroxyflavones; (B) onion skin extract; (C) wine; (D) plasma spiked with quercetin. Peaks: 1, rutin; 2, myricetin; 3, fisetin; 4, morin; 5, quercetin; 6, kaempferol. Structures given in Figure 3. Reproduced with permission from Tsuchiya (1998).

It was noted that the absolute recoveries of quercetin, fisetin and rutin were better than those for the other compounds, and it was suggested that this could be explained by differences in the boronate complexes formed by 1,2 as opposed to 1,3 diols.

Ecdysteroids The ecdysteroids are the moulting hormones of insects and crustaceans. They are relatively polar polyhydroxy steroids (the structure of 20-hydroxyecdysone is given in **Figure 5**) which are widely distributed in nature. Indeed, over 250 ecdysteroids have been isolated from various sources, particularly plants, where they probably function as chemical defences against predatory insects. Many of the ecdysteroids contain one or more vicinal diols, most often encountered at C-2 and C-3 of the A ring of the steroid nucleus, and in the side chain at C-20 and C-22. PBA has been found to provide the basis for the selective extraction of those compounds in possession of a C-20,22 diol group, but not ecdysteroids containing only a C-2,3 structure.

In this instance the extraction of the C-20,22 diol-containing compounds involved activation of the PBA with ethanol (5 mL) and then an alkaline buffer (5 mL, *c.* pH 8). Borate (100 mmol L^{-1} pH 8.0 or pH 8.2) or phosphate (100 mmol L^{-1}, pH 8.0) buffers gave essentially the same result. Under these conditions compounds such as ecdysone and 2-deoxyecdysone, which lack the C-20,22-diol, were extracted from the matrix but were readily recovered using alkaline methanol (70% methanol). The C-20,22-containing compounds were, in contrast, surprisingly well retained and even eluents composed of 90% methanol–1% trifluoroacetyl failed to recover more than 20% of these substances. Quantitative recoveries of these strongly adsorbed ecdysteroids required buffers that contained either salicylic acid (25 mmol L^{-1}) or lactic acid (3% w/v) in 50–70% methanol. A typical chromatogram for 20-hydroxyecdysone from a plant extract is shown in Figure 5.

These differences in the extraction properties of the C-20,22-diol and the C-2,3-diol-containing ecdysteroids is interesting and may well result from the difference in the O–O atomic distances in the two structures. Thus, with the C-2,3 compounds, this distance is 28 pm but with the C-20,22 structures this narrows to 25.2 pm. It is thus possible therefore that a rigid 2,3-diol would be unable to form a cyclic boronate whereas with the less rigid C-20,22 a cyclic boronate is possible.

Drugs and Metabolites

β-Blockers

PBA has been used to extract β-blockers (a class of aminoalcohol-containing drugs) from aqueous solution, rat, and human plasma. The analytes included propranolol, epanolol, ICI 118551 and practolol (see structures in inset to **Figure 6**). The cartridges (100 mg PBA) were first conditioned with 1 mL methanol followed by 5 mL of glycine buffer, following which SPE was performed using 0.1 mol L^{-1} glycine buffer at pH 8.2. Following sample application, nonspecifically retained substances were removed by washing with 1 mL of deionized water followed by 3 mL of methanol–water (40 : 60 v/v). The analytes were then recovered in 3 mL methanol–water trifluoroacetyl (50 : 50 : 1 v/v). The extraction was pH-dependent, with the greated

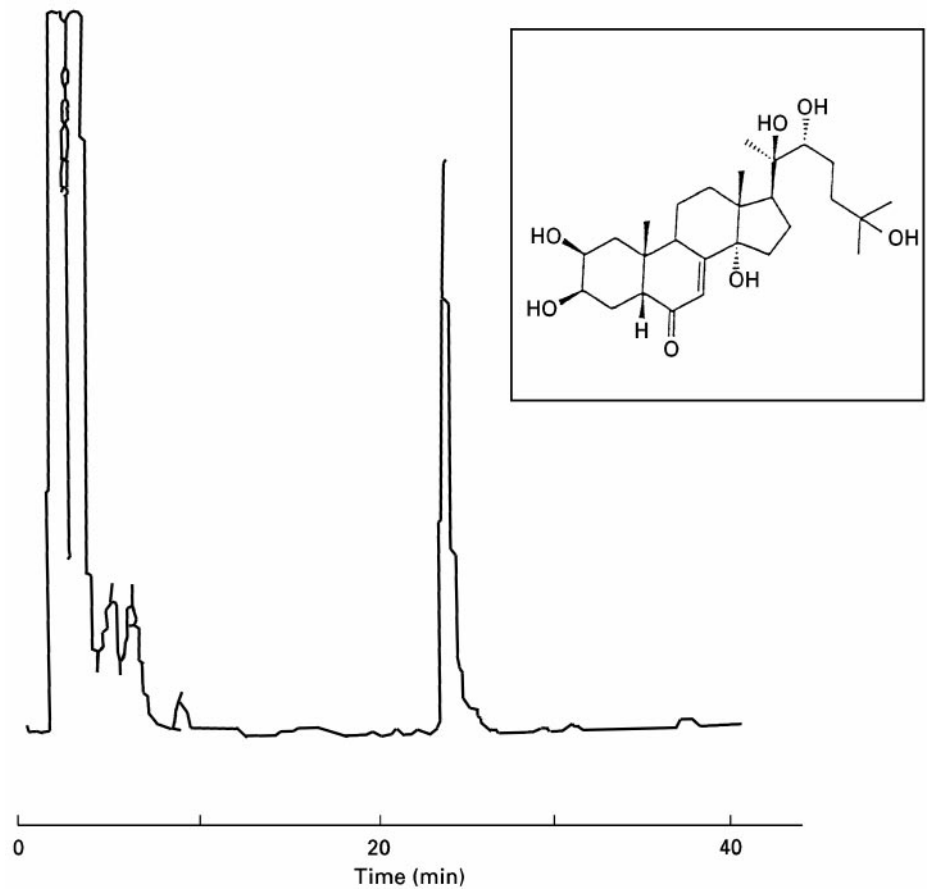

Figure 5 A typical chromatogram obtained for a PBA extract of a plant sample containing 20-hydroxyecdysone (see inset for structure).

extraction efficiency observed at pH 8 (Figure 6) but, in addition, structural features were also important. The extraction was most efficient for propranolol and ICI 118,551 (greater than 90%) with only small losses at the application and wash steps. With practolol, losses at the application step were high ($>7\%$), and both practolol and epanolol showed losses at the wash step (8.8 and 16.5% re-

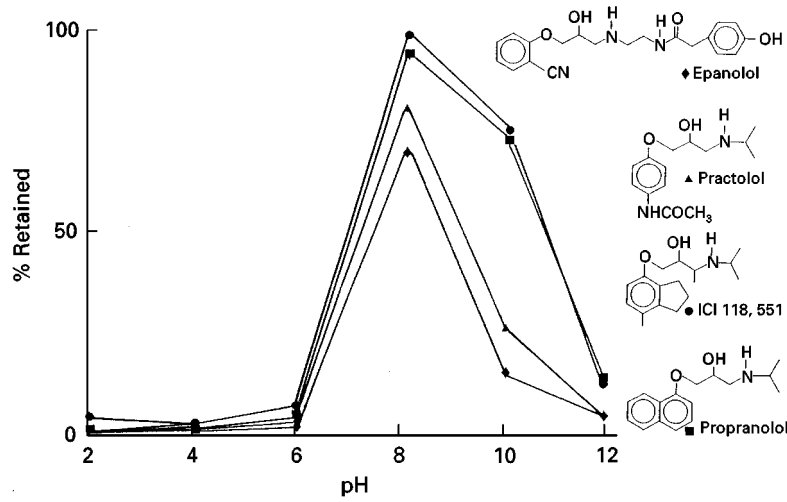

Figure 6 The effect of pH on the extraction of the β-blockers, propranolol, epanolol, ICI 118,551 and practolol on to PBA.

spectively). The latter could be reduced by decreasing the proportion of methanol in the solvent used for elution.

Some matrix effects were also noted for epanolol (but not propranolol, practolol and ICI118,551) when extraction was performed from rat plasma where losses at the application and wash steps were greater than from buffer. This effect, which probably resulted from protein binding, was reduced by diluting the sample with glycine buffer prior to extraction.

Glucuronides

Glucuronides are an important class of metabolites for xenobiotics such as drugs. In many analytical methods these conjugates are hydrolysed back to the aglycone followed by extraction. However, the glucuronides have the potential for SPE on PBA, enabling glucuronide-specific assays to be developed. A limited number of studies into the potential of this type of SPE using a range of model phenolic glucuronides, spiked into urine, have been performed.

The test analytes in these studies were phenolphthalien glucuronide, *p*-nitrophenylglucuronide, α-naphthylglucuronide and 6-bromo-2-naphthylglucuronide present in human urine at a concentration of 5 mmol L^{-1}. The extraction protocol developed for these compounds involved mixing 500 μL urine with 1.5 mL of 100 mmol L^{-1} 8.5 glycine buffer (pH 8.5) which was then applied to a PBA column that had been conditioned first with pH 10 glycine buffer (5 mL, 100 mmol L^{-1}) and then equilibrated with a further 5 mL glycine buffer at pH 5. Glucuronides that were retained on the PBA were then eluted with 5 mL of methanol–1% HCl (90 : 10 v/v). With this protocol the selective retention of some of the test compounds was demonstrated depending upon the structure of the analyte and the amount of PBA employed. With phenolphthalien glucuronide, good extraction was obtained with 300 mg PBA and complete extraction was demonstrated with cartridges containing 400 mg. Extraction on 100 and 200 mg cartridges was, however, incomplete. Good recoveries were seen in the methanol–HCl elution step. Phenolphthalien glucuronide and phenolphthalien sulfate were readily separated from each other using PBA. Good results for the extraction of 6-bromo-2-naphthyl-β-D-glucuronide were also obtained on 600 mg cartridges.

However, with both *p*-nitrophenol glucuronide and α-naphthylglucuronide extraction efficiency was not as good (20 and 50% respectively with 500 mg PBA cartridges).

Thus, whilst it was possible for certain structures to extract phenolic glucuronides on to PBA, and selectively to fractionate sulfates and glucuronides, the presence of glucuronic acid is not of itself sufficient to ensure extraction. Indeed, glucuronic acid itself was not retained under the extraction conditions used for the conjugates. Clearly the structure of the aglycone on to which the glucuronic acid is attached is also important. The exact structural features that would ensure good extraction of glucuronides have still to be elucidated, but π–π interactions may be important. It should also be noted that, whilst glucuronides possess vicinal diol groups with which to form boronate esters, it is also possible that the carboxylic acid and its adjacent hydroxyl group are responsible for the observed extraction.

Miscellaneous

Alizarin The tricyclic anthraquinone dye alizarin contains a *cis*-diol and has been used as a model compound for studying SPE with PBA. The cartridges were conditioned with methanol and then pH 8.6 HEPES buffer (0.1 mol L^{-1}). An aqueous solution of the dye (0.1%) was quantitatively retained, as a sharp band on the cartridge with elution subsequently achieved with methanol–HCl (0.1 mol L^{-1}) (3 : 1). Efficient extraction was only achieved at pH 7–10; however it was also shown that good extractions were also possible from solutions containing up to 70% of an organic solvent as long as they were alkaline. Indeed, as long as wash solvents were alkaline, it was possible to use solvents such as methanol, ethanol or acetonitrile without loss of the dye from the cartridge. The extraction of the analyte from plasma was only efficient if the sample was first extracted on to a C_{18} phase. Elution from C_{18} with an alkaline methanol buffer on to the PBA then resulted in good recoveries. Presumably protein binding was responsible for the poor result (see the β-blockers above).

Conclusions

For those compounds with the structural features that permit it, the possibility of selective extraction using immobilized phenylboronic acid may have potential benefits. Such a sorbent clearly has the potential to result in a relatively specific clean-up and it is perhaps surprising that there are relatively few published applications. However, it is evident from some of the examples provided above that the suitability of a particular analyte for extraction on to PBA can only be determined by experiment as the apparent possession of a suitable structure for cyclic boronate formation (e.g. a *cis*-diol) is no guarantee of success.

See also: **II/Affinity Separation:** Immobilized Boronates and Lectins. **Chromatography: Liquid:** Derivatization. **Extraction:** Solid-Phase Extraction. **III/Ecdysteroids: Chromatography. Appendix 1/Essential Guides for Isolation/Purification of Drug Metabolites.**

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