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An investigation of the properties of a "shielded" phase for the solid phase extraction of acidic and basic compounds

P. Martin[a,*], E.D. Morgan[b], I.D. Wilson[a]

[a]*Dept. of Safety of Medicines, Zeneca Pharmaceuticals plc, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK*
[b]*Dept. of Chemistry, University of Keele, Keele, Staffordshire, ST5 5BG, UK*

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Abstract

The extraction properties of Hisep, a novel bonded phase for solid phase extraction, have been investigated using a number of acidic and basic analytes and compared with those of a conventional ODS bonded material. Marked differences in extraction properties for the test analytes propranolol, anisic acid and ICI 128436 from aqueous buffer were noted between the two materials. Thus, extraction onto the ODS material was by both ionic and reversed-phase mechanisms whilst retention on the Hisep phase appeared to be mediated predominantly by reversed-phase hydrophobic interactions. The presence of dog plasma had no effect on the extraction of propranolol but did reduce the extraction efficiencies observed for anisic acid and ICI 128436 in a pH dependent manner.

Keywords: Solid phase extraction; Propranolol; Anisic acid; ICI 128436; Novel bonded phases

1. Introduction

Solid phase extraction (SPE) is an increasingly popular and important means of preparing samples for analysis, as reflected by the large number of publications in this area, see for example Refs. [1–3]. The technique is likely to assume still greater importance with the increasing problem of disposing of waste organic solvent from liquid–liquid extraction methods. Furthermore, the chlorinated solvents used for extraction purposes are becoming increasingly unacceptable environmentally. There are now very many SPE phases available, from a variety of commercial sources, and whilst in general such diversity is to be welcomed it can however also provide a source of difficulty for the analyst. Thus, although the solvents that are used for liquid–liquid extraction rarely differ markedly in their properties from one supplier to another the same is not true for SPE columns. Whilst it is generally accepted that chromatographic phases will vary in their selectivity with manufacturer (and chromatographers take advantage of this fact), the situation is less well defined for SPE. It is often claimed, for example, that a

* Corresponding author.

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particular C18 SPE bonded phase from one source can readily be substituted for another. The variations in extraction properties between different SPE materials of nominally the same basic chemistry that the present authors [4–6] (and others) have observed have stimulated the development of methods for evaluating new products. With these test systems the aim is first to be able to compare materials from different batches or suppliers, and thus assess their suitability for use in particular applications and, secondly, to gain a better understanding of the selective retention mechanisms of SPE to improve and speed method development. A small number of acidic and basic substances were therefore selected to serve as test compounds with which to evaluate the properties of new SPE materials. Here studies aimed at defining the extraction properties of a newly introduced "shielded phase" for SPE are described and its properties compared with those of a more conventional C18 bonded material.

2. Experimental methods

2.1. Compounds

Three [^{14}C]-radiolabelled compounds were used in these studies: propanolol (2*RS*)-1-isopropylamino-3-(1-naphthyloxy)-2-propanol), specific activity 38.4 μCi mg^{-1}, ICI 128436 (2-(4-bromo-2-fluorobenzyl)-1, 2-dihydro-1-oxo-[1, 4-^{14}C]-4-phthalazinylacetic acid), specific activity 24.4 μCi mg^{-1} (both prepared by Zeneca Pharmaceuticals Radiochemistry Unit), and anisic acid (*p*-methoxybenzoic acid), specific activity 18 μCi mg^{-1} (Amersham International, Amersham, Buckinghamshire, UK) (for structures see insets to Figures). All compounds had a radiochemical purity in excess of 95%.

2.2. SPE methods

The Supelco Hisep cartridges used in these studies (Supelco, Poole, Dorset, UK) contained 300 mg of material. The phase is a silica support with a shielded hydrophobic phase consisting of hydrophobic pockets enclosed by a hydrophilic network (a schematic representation is shown in Fig. 1). This structure is designed to allow penetration of small analytes to the hydrophobic pockets while excluding larger proteins.

The conventional C18 bonded cartridges used for comparative purposes were the Whatman ODS (Whatman, Maidstone, Kent, UK) 100 mg cartridges, with a 5% carbon loading. In order to compare the properties of the Hisep and ODS materials for SPE, the extraction of [^{14}C]-radiolabelled propranolol, ICI 128436 and anisic acid from both aqueous buffer and diluted dog plasma was examined.

The test compounds (at a concentration of 2.5 μg ml^{-1} unless stated otherwise) were loaded on to the cartridges in either aqueous buffers or aqueous buffer containing 25% (v/v) control dog plasma.

The basic extraction protocol for both cartridge types involved solvation with methanol (2 ml), water (2 ml) and an appropriate buffer (2 ml). For pHs from 2 to 5 a sodium citrate buffer (0.2 M) was used whilst for pHs 5 to 10 a sodium acetate buffer (0.2 M) was employed. The sample (either buffer or 25% plasma in buffer; (1 ml) was then applied (at the pH used for cartridge conditioning), followed by an initial wash step with buffer

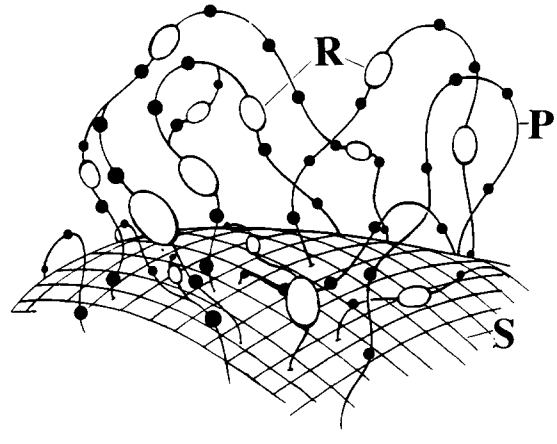

Fig. 1. Diagrammatic representation of the Hisep SPE sorbent: S = silica support; R = hydrophobic pockets; P = hydrophilic network.

(1 ml) and then acetonitrile (0.5 ml). Elution was performed with methanol/0.1M triethylamineacetate (TEA); 80:20 (2 × 1 ml). Variations on this protocol were adopted to investigate specific phenomena as described in the text.

Elution profiles were obtained by extracting samples as described above and then eluting sequentially with methanol–water solutions or methanol–TEA (0.02 M final concentration) of increasing eluotropic strength. For most experiments the eluotropic series employed contained 15%, 30%, 45%, 60%, 75%, 90% or 100% of methanol (v/v). However, in some experiments (see text and Figures) the proportions were changed to 10%, 20%, 40%, 60%, 80%, 90% and 100% of methanol (v/v).

2.3. Scintillation counting

The recovery of radiolabel in the various wash buffers, solvents and eluents was determined using liquid scintillation counting. Eluates from the extraction cartridges were collected directly into 20 ml plastic scintillation vials (Packard), mixed with 10 ml of Ready Value scintillation fluid (Beckman) and analysed on either a Packard TRI CARB 1900 CA or a Beckman LS 1801 scintillation counter with quench correction.

2.4. Sample oxidation

In cases of incomplete recovery of the applied radiolabel the cartridge contents were oxidised to determine the amount of radioactivity retained on the packing material. For sample oxidation the packing was removed from the cartridges and placed in combustacones with two combustapads (Packard) and then oxidised using a Packard 306 sample oxidiser. The radioactive CO_2 produced on combustion was collected into Optisorb 1 CO_2 absorber (LKB) and Optisorb S scintillation cocktail (LKB) and the radioactivity present determined by scintillation counting as described above.

3. Results and discussion

3.1. Extraction of [^{14}C]-propranolol from buffer

To evaluate the properties of the Hisep shielded phase for the extraction of [^{14}C]-propranolol, conditions previously shown to give good extraction onto conventional C18 bonded phases were tried [4–7]. Extraction was examined at pH 5 from aqueous buffer onto Hisep and ODS cartridges prepared by wetting them with methanol followed by water and then buffer as described in the Experimental section. At pH 5 propranolol, a weak base (pK_a 9.42), would be present in the sample entirely in the ionised state. Under these conditions the extraction mechanism for propranolol onto conventional C18 bonded phases is thought to rely on the interaction of the ionised drug with residual silanols on the exposed surfaces of the phase [4]. As expected from previous studies [4–7], extraction of the [^{14}C]-propranolol onto the ODS material was high under these conditions ($> 99\%$). By comparison, extraction onto the Hisep material was poor (59%), with the bulk of the radiolabel eluting in the sample loading (41%) and wash steps (17%). A further 14% of the extracted radiolabel was recovered in the acetonitrile wash. This result was unsurprising, as extraction of the ionised drug would not be expected to occur on a "shielded" material such as the Hisep phase (with restricted access to silanols). That is, where a reversed-phase partition mechanism would be expected to predominate, the ionised drug would not be efficiently retained on the phase. Experiments were therefore performed to examine the effects of pH on extraction efficiency.

3.2. Effect of sample pH on the extraction of [^{14}C]-propranolol from aqueous buffer and dog plasma

The effect of pH on the extraction of the [^{14}C]-propranolol from aqueous buffer and dog plasma was assessed for the Hisep and ODS cartridge types over the pH range 5–10. Since propranolol is basic, below pH 9, where the compound is ionised, extraction via an ion-exchange mechanism onto residual silanols should be favoured.

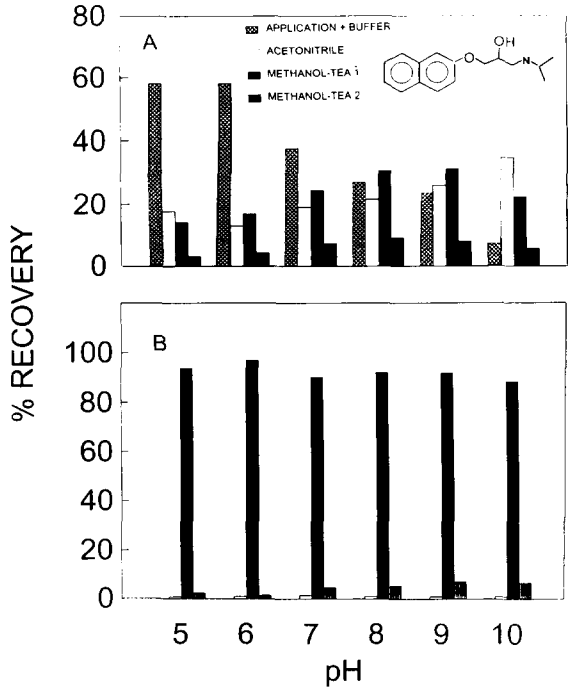

Fig. 2. (A) The pH extraction profile for [^{14}C]-propranolol (structure in inset) following application, wash and elution steps from Hisep SPE cartridges. (B) The pH extraction profile for [^{14}C]-propranolol from Whatman ODS SPE cartridges. Samples were applied in aqueous buffer. For pH 2–5 the buffer employed was 0.2 M citrate, whilst for pH 5–6 0.2 M acetate buffer was used. Key and conditions in inset.

The extraction of propranolol onto the Hisep cartridges from aqueous buffer is illustrated in Fig. 2A. The extraction efficiency was poor at pH 5 and improved with increasing pH up to pH 10. This is the expected behaviour for a reversed-phase, hydrophobic extraction. Whilst extraction efficiency improved as the pH approached the pK_a value of the analyte some losses (7%) were still apparent in the loading and buffer wash steps. The cartridges were then eluted with acetonitrile. The present authors have found acetonitrile to be a very poor eluent for Beta-blockers on ODS phases [4–7], probably because of the inability of acetonitrile to break the strong ionic interactions between such analytes and residual silanols. Indeed, in order to obtain efficient recoveries of such compounds from conventional ODS phases methanol containing triethylamine (as a competing base) had to be used as eluent. Recoveries with acetonitrile ranged from 37% of the adsorbed propranolol at pH 8 to 30% of the adsorbed radiolabel at pH 10 (see Fig. 2A). The bulk of the remaining radiolabel was recovered in subsequent elutions with methanol–TEA. However, even using methanol–TEA complete recoveries of propranolol were not obtained (93% at pH 5 to 85% at pH 10). Unlike the ODS phase (discussed below) the inclusion of TEA was not essential to elute the propranolol from the HISEP cartridges, methanol alone being sufficient to obtain good recoveries.

With experiments on the conventional ODS cartridges very good extractions were obtained at all pH values (> 99.8%). Little loss (< 1%) from the cartridges was seen with the acetonitrile wash, and the bulk (90–97%) of the radiolabel was recovered in the methanol–TEA elution steps (Fig. 2B).

The efficient extraction of propranolol onto the ODS material across the pH range probably indicates that both ionic and hydrophobic interactions are involved, with the balance depending upon the extraction pH. If ionic interactions were solely responsible for extraction onto the ODS phase, extraction would be strongly dependent on pH. Since acetonitrile washes gave a low recovery, irrespective of pH, it would seem that adsorbed propranolol was retained on the ODS material by strong silanophilic, rather than hydrophobic, interactions. Extraction of propranolol onto the ODS material at pH 9 and 10 may therefore need to be considered to be a multistep process, namely extraction of the propranolol from the aqueous phase onto the hydrophobic C18 layer, migration of the propranolol through the C18 layer towards the silanols on the silica surface and then interaction of those molecules in the ionised form with the silanols. As the ionised and unionised forms of the drug would be in equilibrium, removal of the ionised material by the silanols would eventually result in all of the extracted propranolol being retained by the ion-exchange interactions rather than by partition. In this model therefore, extraction and retention need to be considered independently, with extraction seen as a balance between ionic and partition mechanisms, depending upon

the pH, but with retention dependent on ionic interactions with the silanols.

Extraction from buffer solutions represents a rather simple model for sample preparation. The experiments were next extended to the effects of pH on extraction from buffer solutions fortified with dog plasma (25%) in order to determine the importance of matrix effects. For the ODS material, plasma in the sample had little effect on sample extraction efficiency, with less than 2% of the radiolabel being recovered during sample application and was buffer steps. The results for the Hisep phase with plasma buffer generally were similar to those with buffer alone albeit with a tendency for poorer extraction at pH 5 (58% lost on application from buffer vs. 76% from plasma).

3.3. Effect of pH on the extraction of [^{14}C]-anisic acid from aqueous buffer and dog plasma

The extraction of anisic acid was next investigated as such small acidic compounds also form an important class of potential analytes.

The effect of sample pH on the extraction of [^{14}C]-anisic acid from aqueous buffer was assessed over the pH range 2–6 on both Hisep and ODS cartridges. With the Hisep phase, as the pH used for sample application fell below the pK_a of the analyte (pK_a 4) high extraction efficiencies were observed (> 90%). Some losses (8%) were seen at the wash step. However, well-extracted anisic acid was poorly recovered from the Hisep cartridges (≈ 58% of the total extracted) at the elution step (Fig. 3A).

With the ODS phase, good extraction was observed at pH 2 and 3 (> 99%), with much lower extractions obtained at pH 5 (72%) and essentially none at pH 6 (13%) (see Fig. 3B). Such an extraction profile is exactly what would be expected on the basis of retention based on a partition mechanism. At low pH, below the pK_a of anisic acid, the compound would be unionised and thus relatively non-polar and better suited to hydrophobic interactions and reversed-phase extraction.

The effect of inclusion of 25% dog plasma in the sample on extraction of anisic acid at pH 2 and 6 was then investigated. For the Hisep material, predictably, the best results were seen at pH 2, where the matrix had little effect on extraction. At pH 6 this phase was less efficient when dog plasma was present in the sample, presumably in part due to competitive binding to plasma proteins.

The effect of adding dog plasma on the extraction of anisic acid using the ODS material was small at pH 2 and 3. At pH 4, some 30% of the radiolabel was unretained on the column in the presence of plasma compared to complete retention in its absence. At pH 5 and 6 losses at the application step were higher from plasma-containing samples compared to buffer (52% compared to

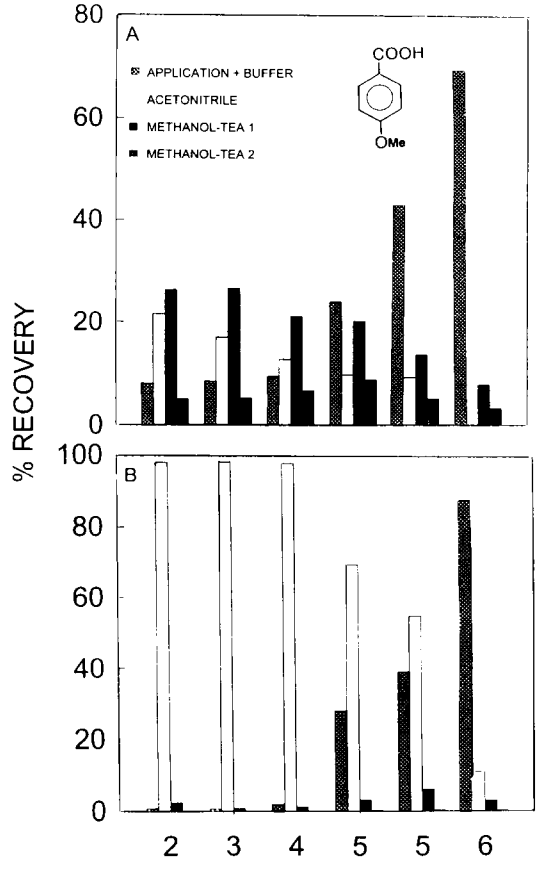

Fig. 3. (A) The pH extraction profile for [^{14}C]-anisic acid (structure in inset) following application, wash and elution steps from Hisep SPE cartridges. (B) The pH extraction profile for [^{14}C]-anisic acid from Whatman ODS SPE cartridges. Samples were applied in aqueous buffer. For pH 2–5 the buffer employed was 0.2 M citrate, whilst for pH 5–6 0.2 M acetate buffer was used. Key and conditions in inset.

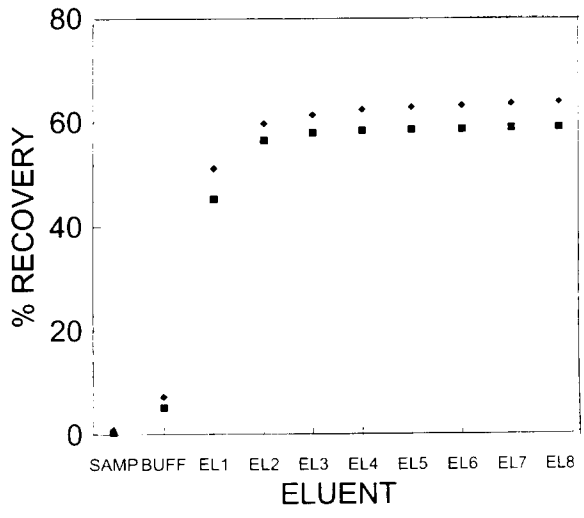

Fig. 4. Cumulative recovery profile for [^{14}C]-anisic acid from Hisep SPE cartridges with successive elutions with acetonitrile (■) or methanol (◆). Samples applied in aqueous buffer (pH 2).

39% at pH 5 and 92% compared to 87% at pH 6). As postulated for the Hisep cartridge, at higher pH the ionised analyte is presumably more subject to protein binding than at pH 2–3, and is less available for extraction.

A puzzling effect of anisic acid on the Hisep phase was the relatively poor recovery at pH values where high extraction efficiencies had been observed. Further studies were conducted using samples extracted at pH 2 (conditions giving complete initial extraction). Once again there was some loss on washing with buffer implying that a proportion of the analyte was only loosely bound to the cartridge. This effect was initially attributed to poor wetting of the phase. However, increasing the amount of methanol used to condition the phase, up to 15 ml, failed to improve the result. Successive washes of the column with acetonitrile or methanol still failed to give complete recovery of the adsorbed anisic acid. The cumulative elution profile for [^{14}C]-anisic acid in these experiments is shown in Fig. 4. Moderate recovery of radiolabel was seen with the first aliquot of either methanol (51%) or acetonitrile (45%), but this fell off rapidly with subsequent washes, and only 59% (methanol) and 64% (acetonitrile) of the total radioactivity could be recovered. The cartridge packing material was therefore removed and oxidised to determine how much of the radiolabel remained adsorbed to the phase. All of the unrecovered radioactivity (approximately 40%) was found to be still present on the Hisep packing.

There is therefore a strong contrast between the behaviour of [^{14}C]-anisic acid on the Hisep material and the ODS phase, where extraction from the buffer was complete. The cause of the poor recovery of anisic acid from the Hisep cartridges is presumably due to the analyte either binding strongly to, or becoming trapped in, the highly cross-linked bonded phase itself.

The effect of concentration of anisic acid on retention on the Hisep cartridges was then examined. With solutions of anisic acid containing 0.5, 2.5 or 25 μg ml^{-1} no differences in the extraction/elution profiles were observed, suggesting a high capacity for the "binding site".

3.4. Effect of sample pH on the extraction of [^{14}C]-128436 from aqueous buffer and dog plasma

Like anisic acid, ICI 128436 is an acidic compound and similar extraction properties were expected. The extraction efficiency of [^{14}C]-128436 from aqueous buffer with Hisep cartridges (Fig. 5A) over the pH range 2–5 was high (> 95%). Losses at the application step then increased gradually from 11% at pH 6 to 32% at pH 10. This pH-related retention is consistent with a reversed-phase mechanism of extraction with higher extraction seen with the compound in the unionised state and losses increasing as the application pH was increased. In contrast to anisic acid, overall recoveries were high on successive elution with either acetonitrile (96%) or methanol (94%). The molecules of ICI 128436 are much larger than those of anisic acid and it may be that they are too large to become "trapped" in the crosslinked phase. The elution profile however changed with pH (Fig. 5A), with acetonitrile eluting the bulk of the radiolabel at pH 2–4, but methanol becoming the dominant elution solvent at pH 5 and above. These data suggest that the retention mechanism for ICI 128436 changes with pH.

The ODS phase similarly gave high initial extraction and subsequent recovery of ICI 128436 from buffer. However, the difference in elution profile with pH seen with the Hisep cartridges was even more pronounced (Fig.5B). High extraction efficiencies were obtained at pH 5 and 6, with the bulk of the extracted ICI 128436 only being recovered in the methanolic eluates (85%). However, ICI 128436 did not behave under these conditions as a simple acid, being efficiently extracted from buffer over the pH range 2–10. The extraction and elution behaviour of ICI 128436 is illustrated in Fig. 5B for pH 2–6. The elution profile obtained over the pH range 6–10 was the same as that obtained at pH 6. Thus the extraction and elution properties of ICI 128436 for pH 5–10 were much more like that of the basic drug propranolol rather than an acidic compound such as anisic acid.

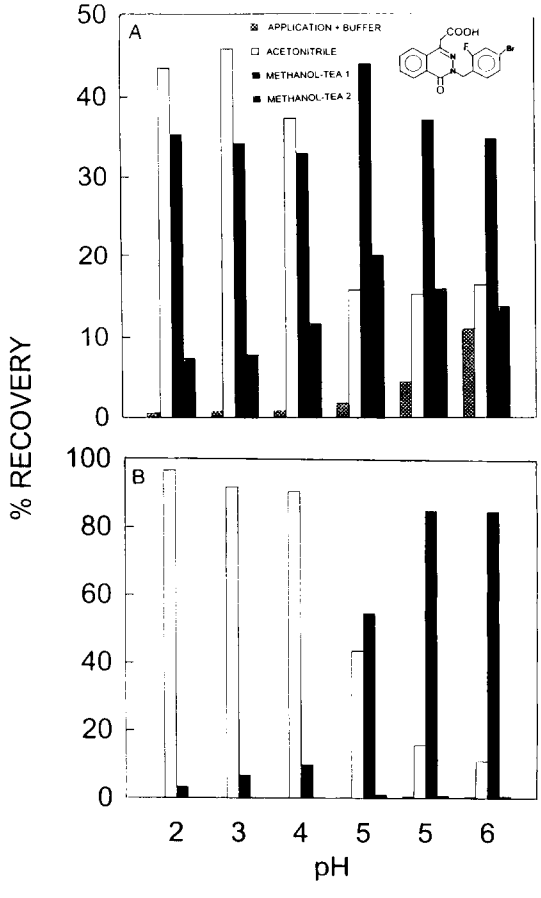

Fig. 5. (A) The pH extraction profile for [^{14}C]-ICI 128436 (structure in inset) following application, wash and elution steps from Hisep SPE cartridges. (B) The pH extraction profile for [^{14}C]-ICI 128436 from Whatman ODS SPE cartridges. Samples were applied in aqueous buffer. For pH 2–5 the buffer employed was 0.2 M citrate, whilst for pH 5–6 0.2 M acetate buffer was used. Key and conditions in inset.

The behaviour of ICI 128436 on both the Hisep and ODS phases is clearly unusual. Ionic interactions with silanols, postulated for the retention of propranolol, cannot readily be invoked in this case, but ICI 128436 is a strong hydrogen bond acceptor and it seems more likely that hydrogen bonding with the silica surface is responsible for the unusual behaviour of ICI 128436.

The presence of 25% plasma markedly reduced the extraction efficiency of ICI 128436 at pH 2 and 6 from the Hisep phase. This effect was greatest at pH 6 where 60.5% of the radiolabel was lost on application in plasma compared to 11% in buffer (at pH 2 the corresponding figures were 37.4 and 0.5% respectively). For the ODS cartridges a reduction in extraction efficiency was seen in the presence of plasma at both pH 2 (18.6% loss on application compared to complete extraction) and 5 (30.7% loss from plasma compared to complete extraction from buffer). These results are probably due to protein binding which may be expected to be enhanced with the analyte in the ionised form.

3.5. Elution recovery profiles

The eluotropic strengths of solvents required to recover the analytes from the two phases were also compared, using conditions chosen to ensure maximum extraction of the analyte from the buffer and then eluting the cartridges with solvents containing increasing proportions of organic modifier (methanol–water or methanol–TEA). Propranolol was therefore extracted on to the Hisep cartridges at pH 10. Elution with increasing amounts of either methanol–water or methanol–TEA (pH 7) gave a rather shallow elution profile (Fig. 6A) and a high organic modifier content would be needed to recover the analyte quantitatively in a small volume. [^{14}C]-Propranolol gave poor recoveries from Whatman ODS (4%) in the

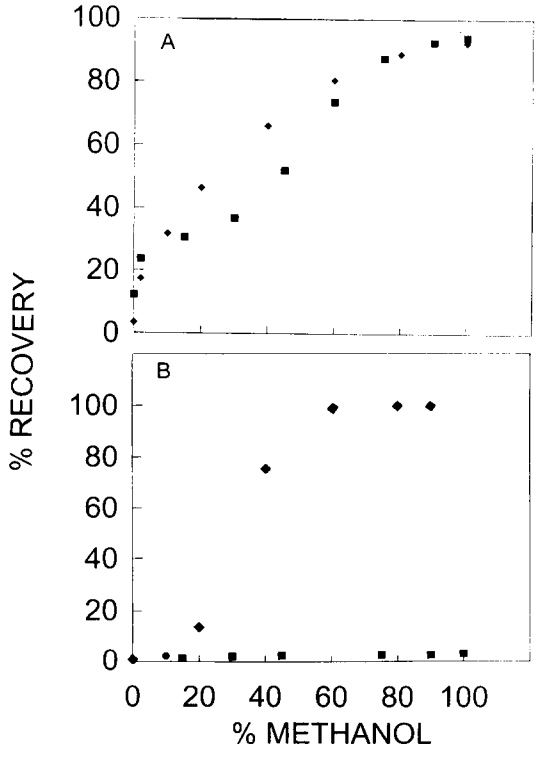

Fig. 6. (A) Cumulative recovery profile for [^{14}C]-propranolol from Hisep SPE cartridges with increasing proportions of methanol (■) or methanol–TEA (◆). (B) Cumulative recovery profile for [^{14}C]-propranolol from Whatman ODS SPE cartridges with increasing proportions of methanol or methanol–TEA.

absence of TEA (Fig. 6B) at up to 100% methanol. With TEA in the eluent, recovery of [^{14}C]-propranolol from the ODS was readily achieved, beginnng at 20% methanol, and was essentially complete with 40% of the organic modifier.

The recovery of anisic acid from the Hisep cartridges also showed a shallow elution profile (Fig. 7) and was little affected by the addition of TEA. The overall recovery of the radiolabel was low (45% using 100% methanol).

Anisic acid was not strongly retained by the ODS phase, some of it eluting with as little as 5% of methanol, and recovery was essentially complete with 15% methanol (Fig. 7).

In contrast to anisic acid, elution of ICI 128436 from Hisep only began when the methanol content reached ≈ 60% (Fig. 8). ICI 128436 was also more strongly retained than anisic acid on the Whatman ODS cartridges, but elution was appreciable at 30% methanol and ≈ 96% with 45% methanol (Fig. 8).

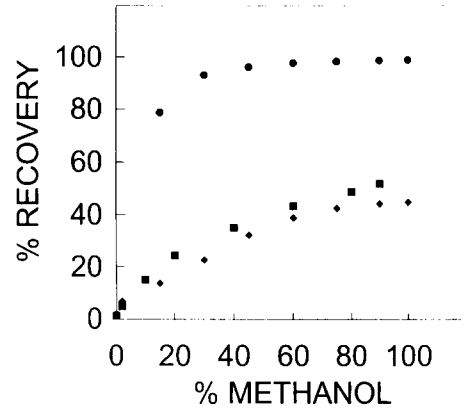

Fig. 7. Cumulative recovery profile for [^{14}C]-anisic acid from Hisep and Whatman ODS SPE cartridges with increasing proportions of methanol (Hisep (◆): Whatman ODS (●)) or methanol–TEA (Hisep (■)).

4. Conclusions

These experiments have revealed major differences between the extraction and elution properties of Hisep and a conventional C18 SPE product. Extraction of propranolol on the C18

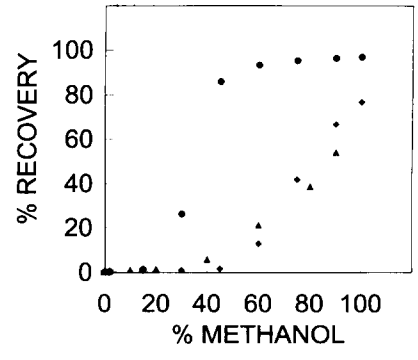

Fig. 8. Cumulative recovery profile for [^{14}C]-ICI 128436 from Hisep and Whatman ODS SPE cartridges with increasing proportions of methanol (Hisep (◆): Whatman ODS (●)) or methanol–TEA (Hisep) (■)).

bonded phases appears to be a combination of reversed-phase and ionic interactions and efficient extraction is possible in the pH range 5–10. In contrast, extraction using Hisep cartridges was poor at pH 5 and improved with increasing pH. This pH-related behaviour was consistent with a reversed-phase extraction mechanism which is favoured with the analyte in the unionised state. The elution profiles for propranolol on the Hisep and ODS phases using acetonitrile support this view. Acetonitrile is a poor elution solvent for compounds retained predominately by ionic interactions, as seen with the ODS phase. In contrast significant, albeit incomplete, elution of propranolol was achieved with acetonitrile from the Hisep phase. No significant matrix effect on extraction was observed for propranolol with the Hisep or ODS phases.

Judging by its pH extraction profile anisic acid behaved as a typical acid on both Hisep and ODS phases. At pH 2–3 the unionised compound was well extracted: as the pH was raised above the pK_a value of the compound, extraction efficiency of the increasingly ionised anisic acid decreased. Overall recovery of anisic acid was atypical on the Hisep phase and never exceeded 70% (despite exhaustive washing with solvents). Plasma adversely affected extraction of anisic acid on to both Hisep and "ODS" phases at pH 6 but not at pH 2, presumably due to protein binding.

ICI 128436 also behaved as a typical acid in its pH extraction profile with the Hisep phase. Below its pK_a value the compound was well retained. At higher pH extraction was less efficient. On the ODS material ICI 128436 extracted well across the entire range examined (pH 2–10). This unexpected result is best explained by hydrogen bonding to the silica surface since ICI 128436 is a strong hydrogen bond acceptor. Unlike anisic acid, good overall recoveries were achieved for ICI 128436 on elution from Hisep cartridges. This can be attributed to the greater size of the ICI 128436 molecules which may penetrate less into the hydrophilic phase and therefore are less likely to become trapped. As seen for anisic acid the presence of plasma reduced extraction efficiency for ICI 128436 on both Hisep and Whatman ODS phases, probably because of protein binding.

It has been found that the Hisep phase possesses extraction properties markedly different from a conventional ODS phase. Novel approaches to the preparation of SPE materials are clearly valuable in providing a different range of extraction selectivities. However, the Hisep phase has some disadvantages for some of the analytes examined, and further changes in its chemistry may be needed to eliminate these.

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