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Use of reduced sorbent bed and disk membrane solid-phase extraction for the analysis of pharmaceutical compounds in biological fluids, with applications in the 96-well format

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Abstract

Significant improvements in the isolation of pharmaceutical compounds from plasma, serum and urine, have been achieved using ultra low mass sorbent bed and thin disk solid-phase extraction (SPE) material. The use of low sorbent masses or disk SPE material has allowed a significant reduction in solvent usage and extraction times. The reduction in solvent volumes required has allowed elution volumes to be reduced to as low as 30 μl with high and consistent analyte recovery. Several SPE RP-HPLC methods have been developed using these materials, including LC–MS methods. When the chromatographic conditions allow the eluent to be injected directly or injected after dilution with distilled water Empore disks are the extraction media of choice due to the materials low elution volume requirements. When operated in the 96-well microtitre format this micro-extraction provides a very efficient throughput and requires little sample manipulation.

Keywords: Solid-phase extraction; Lamivudine; Fluticasone propionate; GM193663

1. Introduction

Liquid–liquid and solid-phase extraction (SPE) methods are used routinely for the isolation of drugs and their metabolites from biological fluids, e.g. serum, plasma, urine and bile [1–4].

SPE involves the use of a solid support such as alkyl bonded silica gel. Solvents and samples are drawn through the packing using either vacuum or positive pressure. The support material is primed with organic and aqueous solvents before the biological fluid is applied, either neat or diluted with buffer prior to application. After sample application the phase is then washed with appropriate solvents to remove any interfering endogenous material, before being eluted with the appropriate solvent, e.g. methanol, acetonitrile or organic acids/bases, into a collection tube.

In liquid–liquid extraction compounds are extracted by virtue of their lipophilicity alone. SPE allows a wider variety of factors to be used, such as polarity, ionic charge, as well as lipophilicity. Thus the isolation of polar compounds and often highly polar drug metabolites from biological matrices is more readily achieved using SPE than is possible with liquid–liquid extraction. SPE also provides the

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opportunity to wash the immobilised sample to remove endogenous compounds. This process can enhance limits of detection by removing co-eluting interfering compounds as well as reducing analytical run times by removing compounds strongly retained on the analytical chromatographic system. SPE cartridges, which are typically syringe barrel, are very amenable to automation [5] and robotic sample processing [6]. By contrast while it is possible to automate liquid–liquid extraction it does require specialised expensive robotic equipment to fully exploit its potential.

The need to quantify both parent drug and metabolites in matrices at low concentrations has necessitated the use of mass spectrometric (MS) detection for GC and HPLC. In LC–MS the two most common modes of ionisation are electrospray and atmospheric pressure chemical ionisation (APCI). In these modes the signal response is greatly increased if the organic solvent concentration of the mobile phase is kept as high as possible and the eluent flow-rate is kept below 200 $\mu l\ min^{-1}$ for electrospray in particular. This low flow-rate either requires splitting a large proportion of the eluent to waste with a 4.6-mm I.D column or using a narrow bore column with an internal diameter of 2 mm or less, thus reducing the flow-rate below 200 $\mu l\ min^{-1}$.

If the elution volume in the SPE process could be reduced to 100 μl or less, with a high analyte recovery, it would be possible to inject the majority of the eluent onto a narrow bore HPLC column. As electrospray gives an increased response with a high organic composition mobile phase, the use of such a phase will allow the injection of eluents with a high organic solvent composition without compromising the chromatographic performance of the system.

The aim of this study was to investigate how sample preparation could be improved by the use of low bed mass silica sorbents and polymeric disc material as solid-phase extraction media in bioanalysis. Particular attention was paid to the minimisation of solvent usage, analyte recovery and reduction in sample preparation time. The results obtained were compared to standard 50-mg syringe barrel SPE cartridges containing the same extraction media. The extraction format was investigated as well the physical dimensions of the extraction bed.

2. Experimental

2.1. Chemicals

Lamivudine, fluticasone propionate and GM193663 were synthesised by GlaxoWellcome (Greenford, UK). Methanol (HPLC grade), acetonitrile (HPLC grade) ammonium acetate (Analar grade) and glacial acetic acid (Analar grade) were purchased from Fisons (Loughborough, UK). Triethylamine (sequanal grade) was purchased from Pierce and Warriner (Chester, UK). Trifluoroacetic acid was purchased from Sigma–Aldrich (Poole, UK).

2.2. Instrumentation

The chromatographic analyses were performed using either a HP1090 integrated, pump, autosampler, column oven and detector (Hewlett–Packard, CA, USA), or a Gilson Unipoint HPLC system consisting of two 307 pumps, 233XL autosampler, 402 diluter (Gilson Medical Instruments, France) linked to an ABI 785A UV detector (Applied Biosystems, CA, USA). MS detection was performed on a VG Platform (VG, Manchester, UK). Integration was performed using a MULTICHROM data system Version 2.2 (VG) for UV detection and VG MASSLYNX version 2.1.

2.3. Sample preparation

Sample extraction was performed using either bonded silica 96-well plates Microsep, C_2, C_8, C_{18}, and SCX of various bed masses (Porvair Filtronics, Shepperton, UK) or Empore disk C_2, C_8, C_{18} and SDBRPS (4 $mm\ l^{-1}$ ml) extraction cartridges (3M, St. Paul, MN, USA). The sample preparation using syringe barrel SPE cartridges was carried out on a 50-place vacuum box (GlaxoWellcome Bioengineering). The sample preparation using 96-well SPE plates was carried out on a vacuum manifold (Porvair Filtronics). In all cases experiments were performed with $n \geq 6$.

The solid-phase experiments consisted of priming the phase with methanol followed by water or an aqueous buffer. The solvent was drawn through the phase under vacuum with care being taken not to let

the phase dry out. The sample was diluted 1:1 with the aqueous prime solvent and then applied to the phase and drawn through under vacuum. The phase was washed with an appropriate solvent and finally eluted with various volumes of an appropriate solvent. During the phase evaluation the solvent from each step, following the application of the sample, was collected and analysed for analyte concentration. The percentage recovery, Fig. 4, was compared to the elution volume with the same amount of drug applied to each phase the aim of this figure was to investigate the low elution volumes claims of the Empore disk material, the concentration is irrelevant.

3. Results and discussion

3.1. Packed bed systems

Traditional packed bed SPE columns have a bed volume of 50–500 mg, which provides good sample capacity and extraction efficiencies. When working with toxicokinetic and clinical (plasma, serum and bile) samples, however, the amount of sample received can be as low as 100 μl. In these cases a large sorbent bed capacity is not as important, in fact it may be a clear advantage to have a smaller sorbent bed as this would involve lower prime, wash and elution volumes as well as faster sample preparation times.

The variation of analyte recovery with elution solvent volumes for various sorbent bed masses, from 10–35 mg, was determined. This was achieved by adding a known amount of aqueous drug to each column and eluting each sorbent bed with increasing amounts of elution solvent from 50–1000 μl. The results of this experiment are shown in Fig. 1. The results indicate that with a 10-mg sorbent bed, 75% of the applied drug can be recovered with an elution volume of just 100 μl, whereas with a 35-mg sorbent bed mass an elution volume approaching 500 μl is required to achieve this recovery.

3.2. Disk cartridge SPE systems

Empore disks are a SPE system based upon the use of a thin polytetrafluoroethane (PTFE) sheets, approximately 0.5-mm thick, into which are embedded SPE sorbents such as alkyl-bonded silicas and polymeric phases. These disks are available in the syringe barrel format in three different diameters, 10, 7 and 4 mm, containing 24, 12 and 4 mg of silica respectively. These different diameters are equivalent to 200, 100 and 50 mg of conventional syringe barrel

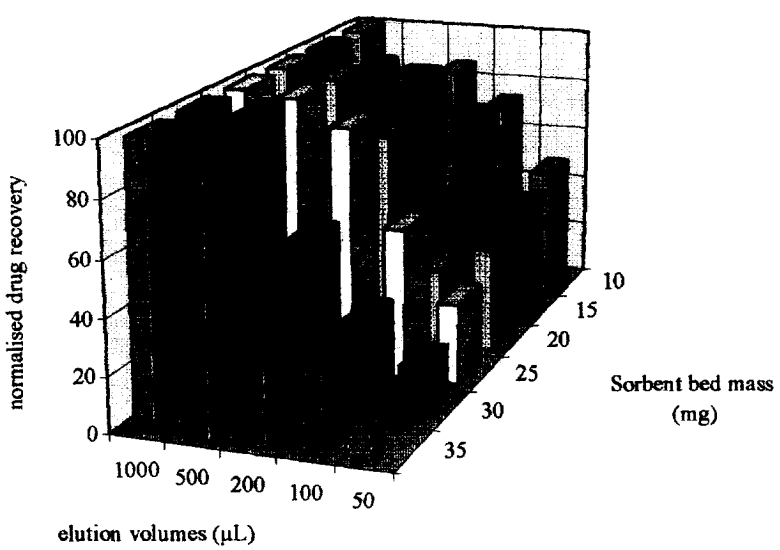

Fig. 1. Comparison of analyte recovery compared to sorbent bed mass with various elution volumes, using the Lamivudine SPE conditions.

loose silica sorbent phase respectively. The analyte recovery with various elution volumes was investigated for the 7- and 4-mm diameter disks and compared to the results obtained for traditional packed bed silica SPE cartridges, Fig. 2.

The results of this experiment show that excellent analyte recovery, 85%, was obtained using the 4-mm diameter Empore disks with an elution volume of just 50 μl. This elution volume of 50 μl for the 4-mm Empore disk is much lower than is required for a standard 50-mg SPE cartridge, thus affording significant savings in solvents usage.

As the SPE material in the Empore disks, is supported by a PTFE membrane, the resulting eluents are totally free from any silica fines thus reducing the possibility of blockages in the chromatographic system. An initial drawback with the use of these disk SPE systems is the low flow-rate produced as a result of the low porosity of the PTFE membrane. This low porosity also requires a high vacuum in order to perform the extraction, typically −1 Bar, which could result in poor flow-rates when applying serum/plasma samples. However, a recent advance in material porosity has been achieved, which has increased the flow-rate without compromising the reduction in eluent volumes. The phase evaluation for the disk material was determined in both the 4-mm syringe barrel and microtitre plate format, the results obtained were similar.

3.3. Analysis time

The ability to reduce volumes used for priming, washing and elution in the SPE process by employing Empore disks or low sorbent bed silica, has also resulted in a significant reduction in sample processing time. The period of time taken for each stage of the SPE process was compared for Empore disks (7- and 4-mm) and traditional silica bed SPE cartridges, Fig. 3.

Using the 4-mm disks the sample processing time can be significantly reduced. Although large savings in time can be made, with the 4-mm disks, in the

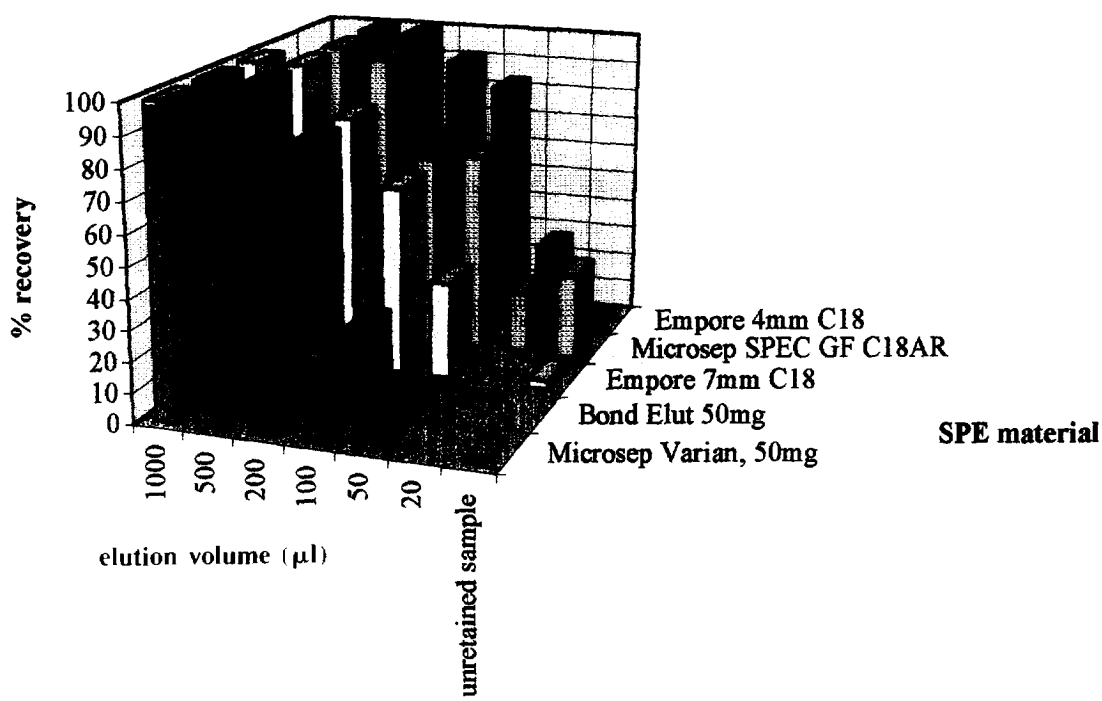

Fig. 2. Comparisons of the analyte recovery with various elution volumes for both disk and packed bed SPE cartridges.

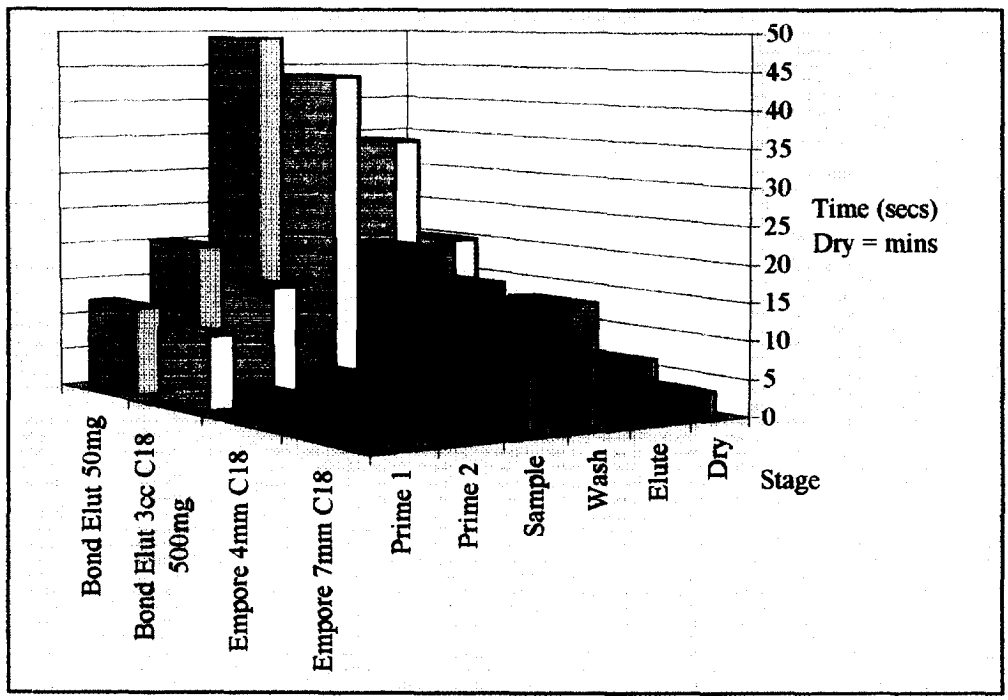

Fig. 3. Comparison of the time taken for each stage of the SPE process using either Empore disks or packed silica SPE cartridges. The volumes of solvent used were optimised for each particular phase. The times are displayed in seconds except for the evaporation (drying time) which is displayed in minutes.

priming, washing, addition and elution stages, the greatest saving in time is achieved in the evaporation stage. The reduction in required elution volume to 50 μl for the 4-mm disks as compared to 1 ml for 50-mg cartridges means that there is a 95% reduction in the amount of solvent to be evaporated. This can result in time saving of between 10 and 20 min per sample. With such a small volume of eluent the possibility arises of directly injecting the eluent onto the chromatography system or diluting the sample with water 1:1 prior to injection, thus completely removing the need for an evaporation step. With a conventional 50-mg cartridge the analysis time for one sample is approximately 20 min, depending on solvent washes, when a 10-mg Microsep system or Empore disk is employed the analysis time can be reduced to just 5 min, including evaporation time. The use of the Empore disk material when combined with the 96-well plate technology can result in a total processing time of just 40 min, when using a multichannel pipette or robotic sample processor, for 96 samples prior to injection.

3.4. Sorbent bed capacity

If the use of Empore disks and low sorbent bed mass SPE is to be adopted in bioanalysis, it is essential to demonstrate that the material has sufficient capacity to accommodate the likely samples concentrations. There must be sufficient for the extraction of the compounds of pharmaceutical interest from up to 500 μl of serum, plasma, urine or bile. The phase capacity was examined for Empore disks (4 mm) as well as 10- and 50-mg sorbent bed mass, by comparing the response of the extracted eluent with the amount of analyte applied (Fig. 4). A vast excess of eluting solvent was employed, and the rate of sample application was kept constant to ensure the study was not biased. These results show that the 4-mm Empore disks and 10-mg SPE cartridges have

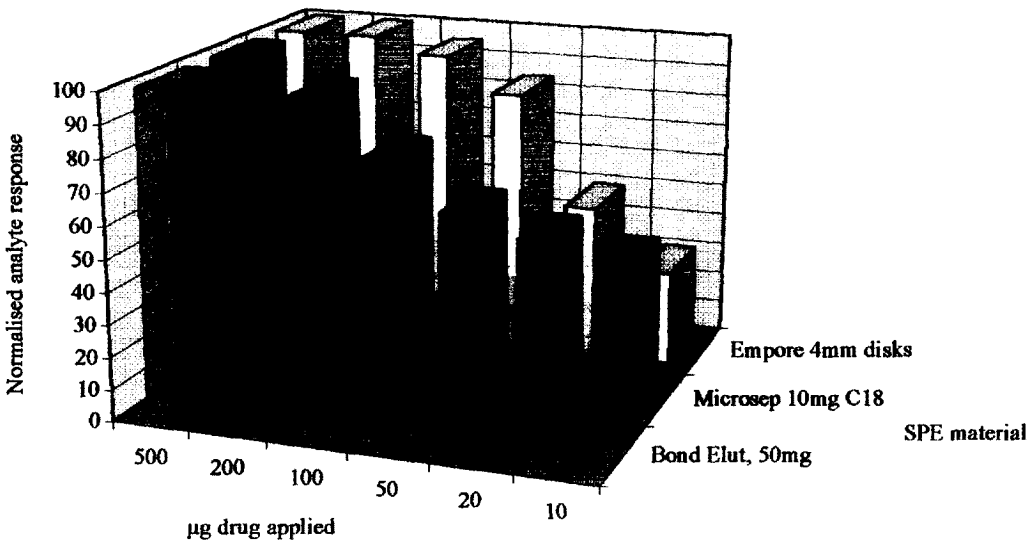

Fig. 4. Comparison of detector response with the amount of analyte applied to the phase.

a maximum loading of approximately 50 µg and 100 µg respectively, whereas the 50-mg phase has a capacity of approximately 500 µg. For toxicological support and initial clinical support work the amount of sample matrix available may be limited to a few hundred microlitres. If the volume of sample applied were to be 100 µl this would mean that analyte concentrations of up to 500 µg ml^{-1} could be handled. In general this capacity will be more than adequate for the low level analytical work involved in drug development as the dynamic range of the assay is not likely to go beyond this, any samples with a concentration greater than 500 µg ml^{-1} would be diluted and reanalysed.

The variation of analyte response with increasing loading of plasma matrix was determined for the 4-mm Empore disks. This was achieved by loading a constant amount of analyte onto the disk whilst increasing the amount of matrix loaded onto the phase from 100 to 1000 µl; the results obtained are shown in Fig. 5. The analyte response was not significantly adversely affected by the amount of plasma loaded on to the phase, thus this phase can be successfully used with an matrix volume of up to 200 µl. This will allow a possible four-fold concentration effect when combined with a 50-µl elution volume.

3.5. The 96-well microtitre plate format

The SPE process has been transferred into the 96-well microtitre plate format. In the 96-well format the microtitre plate wells have an internal diameter of 4.1 mm; this is ideal for use in conjunction with the Empore disks, as 4 mm has been proved to be the ideal disk diameter for bioanalytical work. Traditional SPE uses vacuum boxes which have a capacity of between 20 and 50 cartridges, but the use of the 96-well format has allowed a significant increase in the number of samples that can be processed in each batch, Fig. 6. The Empore disk material and 10, 15 and 50 mg Bond Elute material have been successfully used in the 96-well format, with a resulting increase in analytical throughput. The results from Fig. 2 clearly show that there is no difference between the Varian C_{18} packing in the 96-well plate, Microsep, and the Varian C_{18} syringe barrel cartridge. The low prime, wash and elution volumes feasible with Empore disks and low sorbent mass SPE cartridges have been exploited to reduce sample

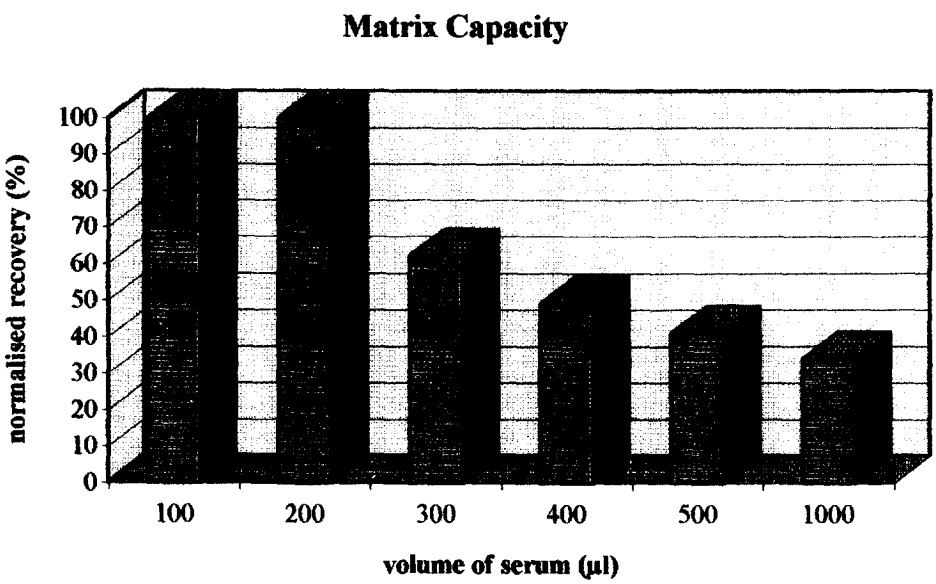

Fig. 5. Effect of an increased loading of plasma matrix on analyte detector response.

preparation times. The Empore disks were fixed into the 96-well plate using a polycarbonate frit as a support above and below the PTFE membrane, the material was placed into each well making a friction fit to the sides of the well. There was no evidence of any material leaking around the junction of the disk and the well wall.

The 96-well format can be operated either manually or with the aid of a robotic sample processor. The 96-well SPE plate is operated in a similar way to the syringe barrel cartridges using a vacuum box. The advantages this format offers is the samples are processed in parallel, rather than in series using a multihead pipette. The eluted solvent is collected in a microtitre plate which can be directly injected from, using a commercially available HPLC autosampler. This can result in a significant cost saving in autosampler vials, caps etc as well as removing the possibility of transfer errors.

We have successfully developed several assays for the determination of drugs and their metabolites from serum and plasma using the 96-well format. The low elution volume of between 30 and 100 µl has allowed, in several cases, the direct injection of the eluent onto the chromatographic system. In other cases simple eluent dilution with distilled water is sufficient prior to injection; this not only eliminates the time required for solvent evaporation but removes any losses due to sample transfer.

3.6. Automation

The 96-well SPE process has been automated within GlaxoWellcome using the Cambara Packard robotic sample processor and a Gilson 233 autosampler [6]. The 96-well format has the advantage of allowing automated parallel processing which significantly increases the throughput. The ability of the Gilson autosampler to inject from a 96-well microtitre plate has removed the need to transfer the SPE eluent to autosampler vials. This not only saves time and eliminates sample loss during the transfer process, but allows the use of low elution volumes e.g. 50 µl, which would not be possible if the sample had to be transferred to an autosampler vial.

3.7. Analysis of analytes in a biological matrix

The low sorbent bed mass SPE cartridges and the Empore disks have been successfully used in the

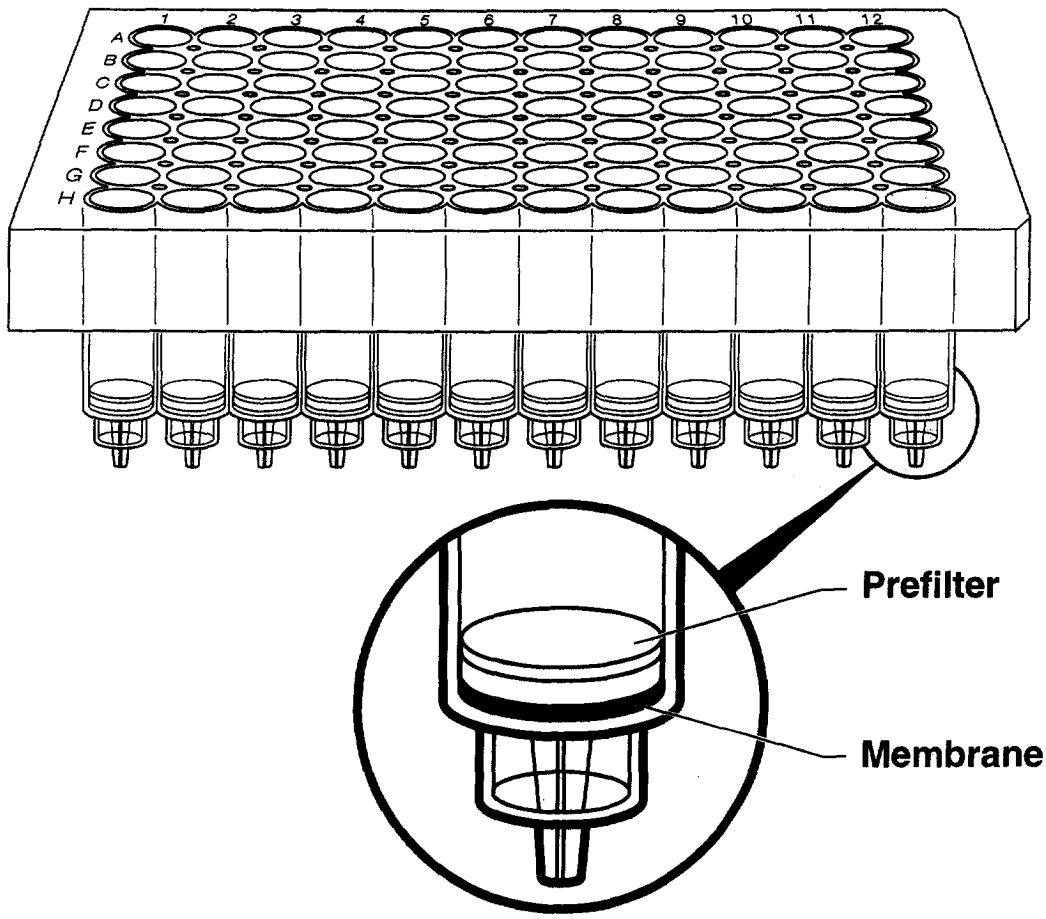

Fig. 6. Example of the 96-well SPE microtitre plate, packed with C_{18} bonded silica Empore disks.

analysis of pharmaceutical compounds in biological matrices. The analytes have ranged in chemical nature from highly lipophilic steroids to nucleoside analogues which are very water soluble. These assays have been used to quantify samples in the low ng ml^{-1} concentration region, and have proved to be both robust and reproducible. The assay for the determination of GM193663 was used to support the drug development program and several hundred samples were analysed using this method, the method was validated for non-clinical samples. Specimen chromatograms for the analysis of fluticasone propionate (a glucoosteroid), lamivudine (a nucleoside analogue) and GM193663 (a tricyclic carboxylic acid) are given in Figs. 7–9, along with the extraction conditions.

4. Conclusion

Empore disks and low sorbent bed mass SPE cartridges have been successfully employed for the analysis of drugs and drug metabolites in biological fluids. The SPE, prime wash and elution volumes have been significantly reduced allowing faster sam-

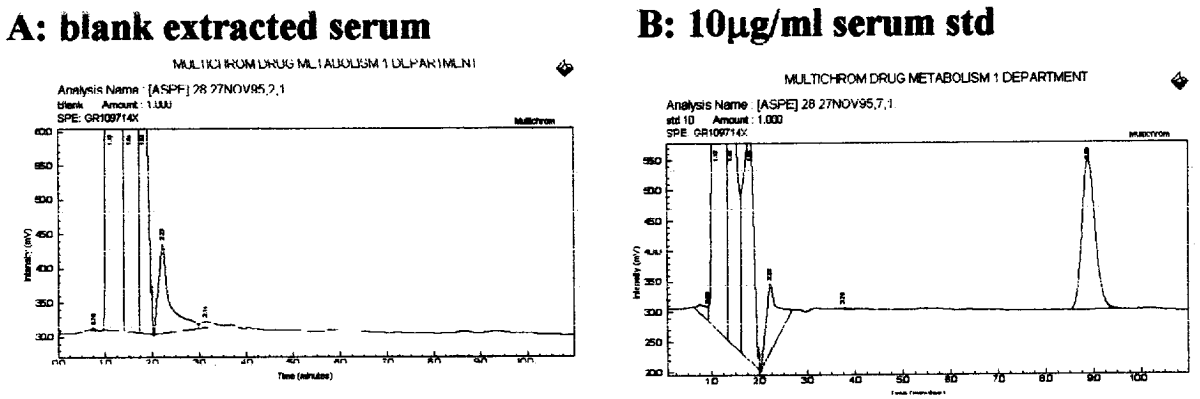

Fig. 7. (A) Extracted blank and (B) 10 μg ml^{-1} fluticasone propionate std. using C_8 10 mg 96-well SPE plate. Chromatographic conditions: column, Supelco ABZ-plus 100×2 mm; mobile phase, acetonitrile–water–formic acid (50:49.5:0.5, v/v/v); column temperature, 40°C; flow-rate, 200 μl min^{-1}; injection volume, 50 μl; detection, UV at 238 nm.

ple processing and increased method sensitivity. The capacity of the Empore disks and low sorbent mass phases have proved to be sufficient for the needs of bioanalysis. When the chromatographic conditions allow the eluent to be injected directly, or injected after dilution with distilled water Empore disks are the extraction media of choice, due the extremely low volume of eluting solvent required. Thus allows all the sample to be directly injected onto the system without an evaporation step. The use of the 96-well format has allowed a significant increase in sample throughput. The 96-well format SPE system has been successfully automated using a Gilson 233 auto-sampler.

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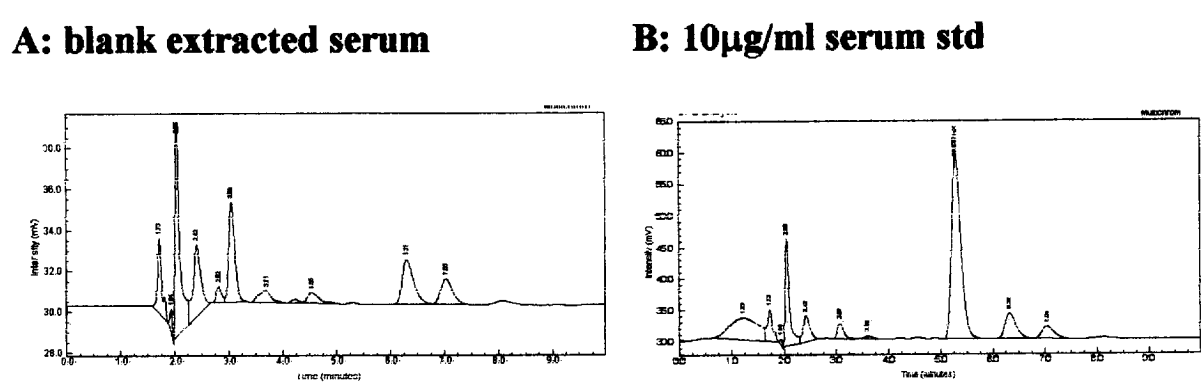

Fig. 8. (A) Extracted blank and (B) 10 μg ml^{-1} lamivudine std., using C_{18} 10 mg 96-well SPE plate. Chromatographic conditions: column, Hypersil BDS 100×2 mm; mobile phase, methanol–0.1 *M* ammonium acetate (10:90, v/v); column temperature, 25°C; flow-rate, 200 μl min^{-1}; injection volume, 50 μl; detection, UV at 270 nm.

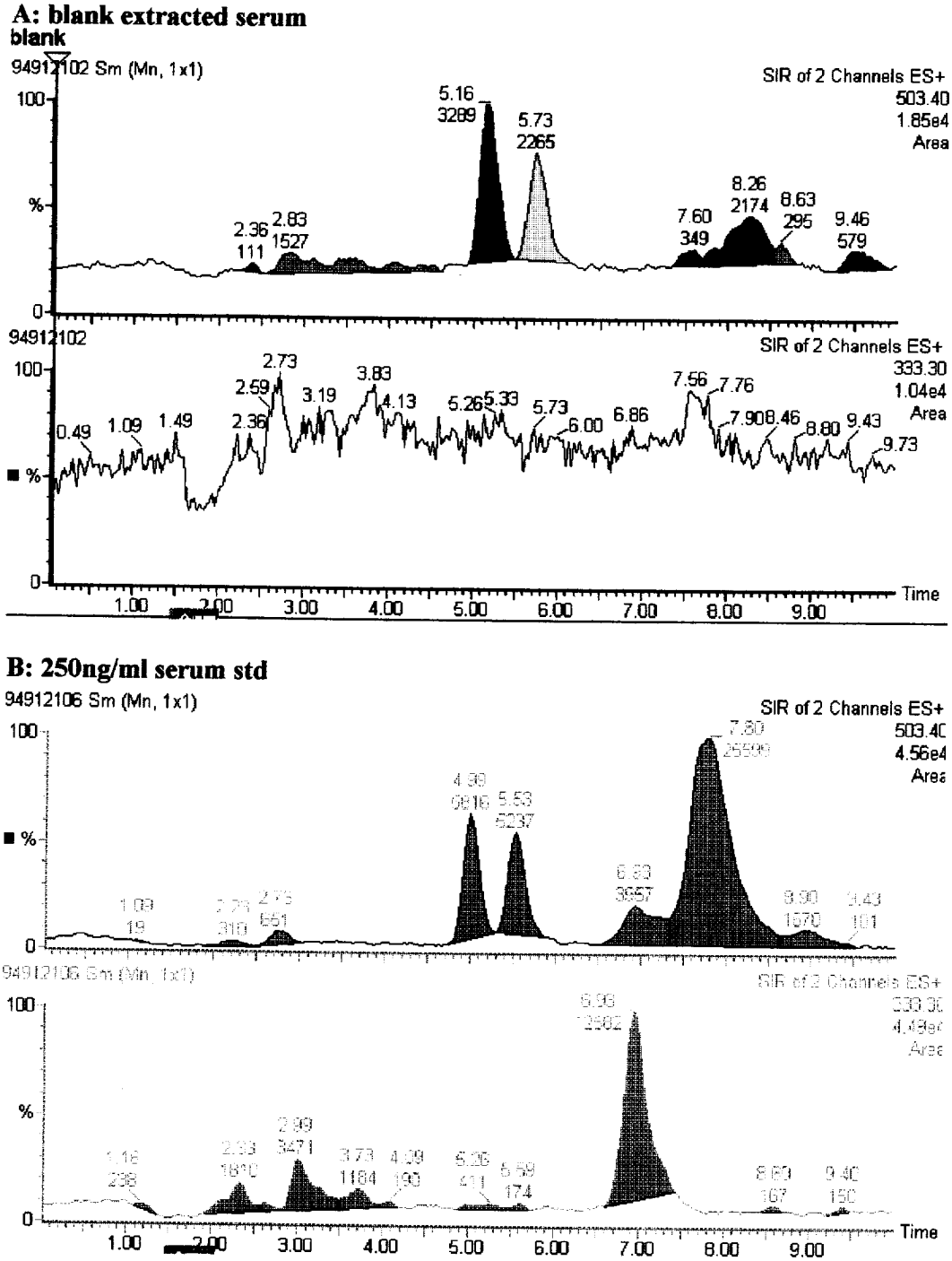

Fig. 9. (A) Extracted blank and (B) 250 ng ml^{-1} GM193663 std., using C_2 Empore 96-well SPE plate. Chromatographic conditions: column, Supelco ABZ-plus 100×2 mm; mobile phase, methanol–water–formic acid (75:24.5:0.5, v/v/v); column temperature, 40°C; flow-rate, 200 μl min^{-1}; injection volume, 50 μl; detection, SIM mass spectrometry. The top chromatogram is the SIM trace for the internal standard, the bottom chromatogram is the SIM trace for GM193663.

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