The determination of drugs, metabolites and endogenous compounds in biological fluids usually involves an isolation procedure prior to the chromatographic step. The most frequently used procedures are liquid-liquid extraction, protein precipitation, and off-line solid phase extraction with disposable extraction columns. All isolation procedures are time consuming and introduce errors into the assay. The more manipulations the samples undergo prior to the quantitation step, the lower the accuracy and precision obtained. Therefore, it is advantageous to inject the sample directly into the liquid chromatographic system without off-line isolation procedures.

It has always been a challenge for the bioanalytical chemist to develop methods where the sample preparation step can be automated and integrated with the LC procedure. Some columns for direct injection of plasma/serum have reached the market, but they have not been designed for optimal performance and are rarely used.

This article describes a technique for online sample preparation where the surface chemistry and hardware design allow injection of large volumes of plasma or serum. By using this specially designed extraction column (BioTrap 500 C18), it is possible to directly inject plasma or serum samples into the LC system. This column allows the separation of high molecular weight compounds from small molecular analytes prior to injection of the analytes onto the analytical column. The BioTrap 500 C18 column is the second generation column based on the same concept and has been described in the literature (1,2 and references therein).

Description of the Online Sample Preparation Technique

The BioTrap 500 C18 column is a small cartridge column (20x4 mm, 13x4 mm or 20x2 mm).

The column is based on silica particles with a biocompatible external surface and a hydrophobic internal surface (C18 groups). The biocompatibility has been obtained by attachment of the plasma protein α1-acid glycoprotein (AGP) on the external surface of the particles. The pore sizes of the particles have been chosen so that plasma proteins and other large molecules will be excluded from the pores (F1).

The cartridge is installed in a 6-port valve between the autosampler and the analytical column as outlined in F2.

Two pumps are used, one for the extraction mobile phase (pump A) and the other for the analytical mobile phase (pump B). The extraction mobile phase is a buffer with a low content of organic modifier, optimized in order to get high recoveries of the analyte and an effective rinsing of the extraction column. The analytical mobile phase is used for the transfer of the analytes from the extraction column to the analytical column and is optimized for the final separation on the analytical column.

The plasma sample is centrifuged and transferred to an autosampler vial. The extraction mobile phase transports the plasma sample from the autosampler onto the BioTrap 500 C18 cartridge. The drugs are trapped on the column during the conditions used in this step, and the plasma proteins are transported to waste. After washing with the extraction mobile phase, the valve is switched to the elution position and the extraction column is backflushed with the analytical mobile phase. The analyte elutes to the analytical column for separation. When the analyte has been transferred to the analytical column, the valve can be switched back to the extraction position for reequilibration with the extraction mobile phase.

The applicability of the online-extraction technique with BioTrap
500 C18 is exemplified in \textit{F3} (carbamazepine and phenytoin), \textit{F4} (propranolol) and \textit{F5} (nortriptyline). These examples show different plasma volumes injected (50 µL, 500 µL, and 200 µL) and different ways of detection (UV and fluorescence).

The Extraction Process

Normally, a very high recovery can be obtained using the BioTrap 500 C18 column. The principle behind the extraction process taking place in the column is outlined below.

A drug (S) in a plasma/serum sample is normally bound to the plasma proteins (P) to a high degree according to the equilibrium below:

\[ P + S = PS \]

where PS is the drug-protein complex. This means that the drug is present in the plasma or serum as free, non-protein-bound drug (S), and protein-bound drug (PS). When such a sample is injected on the extraction column the free non-protein-bound fraction of the drug can penetrate the pores and be adsorbed onto the hydrophobic internal phase of the particles. The drug molecules are trapped if they give sufficiently high retention with the chosen extraction mobile phase (see below concerning the retention in presence of protein in the sample zone).

When the plasma/serum zone continues to migrate on the column, more protein-bound drug is released according to the equilibrium above. The released drug is trapped and the extraction process continues until the plasma/serum zone has been pumped out of the column.

The retention of a drug (S) on the extraction column can be expressed by

\[ k'(S) = \frac{qD(S)}{1 + qD(S)} \]  \hspace{1cm} (1)

where \( q \) is the phase ratio and \( D \) is the distribution ratio of the solute between the solid and the mobile phase. However, injection of plasma/serum samples containing large amounts of proteins, such as albumin and \( \alpha_1 \)-acid glycoprotein (AGP), involved in the binding of drugs and endogenous compounds, affects the chromatographic properties of the low-molecular-mass solutes. Albumin is the drug-binding protein that is present in plasma at the highest concentration and it is involved in the binding of a very broad range of drugs. Albumin has a molecular mass of about 66,000 with the dimensions 150 x 38 Å. The pores of the particles in the BioTrap 500 C18 column have been chosen to exclude the plasma proteins from the internal hydrophobic surface of the particles. If a certain solute (S) is bound to only one binding site on albumin (P), and if the protein is excluded from the pores and the binding of the protein to the external surface of the particles in the extraction column can be neglected, the distribution ratio, \( D \), of the solute (S) can be expressed by

\[ D_s = \frac{[SA]}{[S] + [PS]} \]  \hspace{1cm} (2)

where \( S \) and PS are the concentrations of the free drug and the drug-protein complex, respectively, and SA is the concentration of the drug bound to the binding site, A, on the hydrophobic internal surface of the
Analysis Program:

<table>
<thead>
<tr>
<th>Switching valve pos.</th>
<th>Analysis Step</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extraction</td>
<td>transfer of analyte</td>
<td>5 min</td>
</tr>
<tr>
<td>2. Elution</td>
<td>separation and reequilibrium</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Analytical mobile phase: 28% acetonitrile in 116 mM NaH₂PO₄ pH 2.8
Flow rate: 0.8 mL/min
Fluorometric detection: Ex = 220 nm, Em = 340 nm

In F4 atenolol is extracted and chromatographed using the ion pair technique with BioTrap 500 C18. 200 µL of plasma is injected. If atenolol is extracted on the BioTrap 500 C18 column without the addition of the ion-pair agent, the recovery is around 50%. When 5 mM sodium octanesulfate is added to the extraction mobile phase, the recovery is increased to ~100%.

F7 shows a chromatogram obtained after online extraction of 500 µL of plasma, containing 12 ng/ml of propranolol. In this method 5 mM octanesulfonic acid sodium salt is added to the extraction mobile phase and 2 mM to the analytical mobile phase.

2. The displacement technique

An acid can be extracted in two different ways, as the protonated acid and as an ion pair. Normally a very good recovery can be obtained by extraction of the protonized acid at low pH.

Another way to increase the recovery is to use neutral pH and extract the acid as an ion pair with the positively charged buffer ions like sodium, with a hydrophobic acid (for example octanoic acid) present in the extraction mobile phase, which will compete with the analyte for the protein binding and displace the analyte. This can also have a very positive effect on the efficiency. Furthermore, in this way the peaks can be compressed and the recovery can increase. This is very favorable in low concentration analysis. Such results are shown in F8 for ibuprofen, where 10 µL of serum is injected. The concentration is 20 µL/mL, however, as can be seen from the chromatogram, much lower concentrations can be detected due to the very good chromatographic performance, which means that the method can easily be used for determination of the concentration after single doses of ibuprofen.

Strategies for Increased Efficiency and Recovery When Using BioTrap 500 C18

1. The ion-pair technique

A strategy has been developed to obtain high separation efficiency for basic analytes and to increase recovery for more hydrophilic basic analytes, when using the BioTrap 500 C18 column. These effects are created by the addition of an ion-pairing agent, for example the sodium salt of an alkylsulfonic acid, in the extraction mobile phase. The concentration of the ion-pairing agent is 1 - 10 mM.

In F6 atenolol is extracted and chromatographed using the ion pair technique with BioTrap 500 C18. 200 µL of plasma is injected. If atenolol is extracted on the BioTrap 500 C18 column without the addition of the ion-pair agent, the recovery is around 50%. When 5 mM sodium octanesulfate is added to the extraction mobile phase, the recovery is increased to ~100%.

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Method Development

When developing methods on the BioTrap 500 C18 the same basic principles can be used as in liquid-liquid extraction. However, since the column is silica-based and the sample is plasma or serum, the pH must be in the range that both silica and the sample tolerate, i.e., 4-7.5. The method development strategy is outlined below. It is recommended that one start according to the principles described below since this is a good way to learn how the extraction column works and how it should be used in connection with the analytical column. Subsequent methods will be developed much easier from this basic understanding.

In Fig. 4-7 examples are given for the mobile phase composition and switching times used for representative analytes.

1. Decide how to detect the analyte in order to obtain an adequate detection limit and selectivity. The following detection means are, of course, most favorable in order to avoid disturbing peaks in the chromatogram:
   - fluorimetric detection
   - electrochemical detection
   - mass spectrometric detection
   - UV at a wavelength with high detection selectivity.

   It is sometimes possible to use UV-detection at low wavelengths (210-230 nm) if no interfering peaks are present. Detection at low wavelengths often generates very high sensitivity, but the detection selectivity is very poor.

2. Develop a preliminary analytical method by choosing a column and a mobile phase composition giving good chromatographic performance. This step is performed using only the analytical column.

3. Determine which extraction mobile phase will obtain adequate recovery by using the character of the analyte. It is also important to remember that due to the presence of plasma during the extraction step and the silica base of the extraction column the pH limits of the extraction mobile phase are 4-7.5. An organic solvent can be added to the extraction mobile phase in order to improve the washing of the extraction column. Examples of solvents are 2-propanol (<5), acetonitrile (<10) and methanol (<5).

Basic Analytes

The pH should be adjusted to around 7. If the analytes are positively charged at pH 7, they will be extracted as an ion pair with the anionic component of the buffer, which preferably is a phosphate buffer.

We recommend, however, that the sodium salt of an alkylsulfonic acid be added to the extraction mobile phase in a concentration between 1-10 mM. This hydrophobic counter-ion will give a more effective extraction of the analyte. An example is shown in Fig. 6, where the sodium salt of octylsulfonic acid is added for the extraction of atenolol. In this example both the separation efficiency and the recovery are strongly increased by the addition of the ion-pairing agent. The recovery increases from 50% to ~100%.

Acidic Analytes

If an acid is extracted at a pH which is two pH units below the pKₐ value of the acid, it is extracted as the protonated acid. An acid in its anionic form is extracted as an ion pair with the cationic compound of the buffer, if no other ion-pairing agent is present. To obtain high recovery for an acid, the pH should be as low as possible during extraction. However, to avoid plasma pre-
When using the injection volume that is needed for the analysis.

5. Confirm that the analyte is trapped on the extraction column during the conditions used.

6. Based on steps 4-5, the first switching time can be set, i.e. the time when the valve is switched from the extraction to the elution position. In F4-8 examples are given of switching times for different types of analytes. It is important to be sure that all proteins are eluted from the extraction column before the elution with a mobile phase containing high concentrations of, for example, acetonitrile or methanol is started.

7. Determine the time it takes to complete the elution of the trapped material from the extraction column onto the analytical column using the analytical mobile phase. When this is known, it is possible to determine the second switching time, i.e. the time when the valve is switched back to extraction position and the extraction column is equilibrated with the extraction mobile phase to get ready for the next injection. In F4-7 examples are given for switching times for different types of analytes.

8. Now the extraction column and the analytical column can be coupled together using a 6-port switching valve. The analytical method is optimized using spiked plasma samples. The conditions are adjusted to avoid disturbing endogenous compounds.

9. Validate the method by determining recovery, precision, intraday, and interday variation. The accuracy and the precision in the method will be very high, due to the few manipulations that the samples undergo. Standard curves normally give r-values $\geq 0.9999$.

**Conclusion**

The new BioTrap 500 C18 column enables the development of bioanalytical methods with very high accuracy and precision since there are, in principle, no manual manipulations of the sample. The precision in the injection volume of the autosampler will determine the precision of the method. Furthermore, no internal standard is needed. The serum/plasma samples can be analyzed by LC directly, without prior time-consuming offline extraction (solid-phase extraction, liquid-liquid extraction). The extraction columns can be used for a large number of samples, which together with the time savings, provides good economy. Large serum or plasma volumes can be injected when required. Conventional LC equipment is used, which further keeps the investment low and the reliability high. The BioTrap is quite useful with LC/MS as well as more conventional detectors.

**References**


2. J. Hermansson and A. Grahn. Direct injection of large volumes of plasma/serum on a new biocompatible extraction column for the determination of atenolol, propranolol and ibuprofen. Mechanisms for improvement of the chromatographic performance. Submitted for publication.