BioTrap Sample Extraction Column: A New Approach for On-Line Sample Preparation in Drug Metabolism Studies

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Traditional sample preparation techniques for chromatography of drugs and metabolites in biological samples are tedious and time-consuming. Multi-step pretreatment procedures are required to extract the analyte from the protein matrix. BioTrap 500, a new biocompatible sample extraction column containing porous silica particles, allows direct injection of biological samples into an LC system. The separation of analyte from protein in BioTrap is based on a combination of size exclusion and reverse phase partitioning. In this study the application of BioTrap 500 for on-line sample extraction was demonstrated by investigating the metabolism of testosterone (TTT) to 6β-hydroxytestosterone (OH-TTT) by cytochrome P450 enzymes in rat liver microsomes.

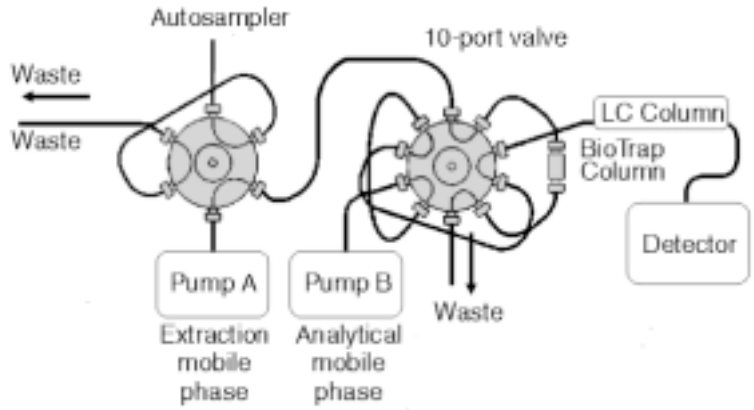
Chromatography of drugs and metabolites in biological samples requires numerous sample preparation steps to extract the analyte from the matrix. Traditional sample preparation involves precipitation of protein by acid or organic solvent and removal of precipitated protein followed by the extraction of the analyte into an organic phase by liquid-liquid or solid phase extraction. These time-consuming sample preparation steps add more complexity to the assay scheme, which results in a decrease in precision and accuracy. A great deal of effort has been directed towards developing new techniques that enable direct on-line injection of samples for analysis.

BioTrap 500, distributed by BAS, is a new, biocompatible sample extraction column containing porous silica particles. The principle of separation of analyte from protein by the BioTrap 500 was described in detail in a previous issue of *Current Separations* (1). Briefly, the separation of analyte from protein is based on a combination of size exclusion and reverse phase partitioning. Small analyte molecules enter the pores and are retained by the C_{18} or C_8 groups on the inner surface. The larger protein molecules are washed away with the continuously flowing extraction mobile phase. After allowing sufficient time to wash the protein off, the valve is switched so that the elution mobile phase can now go through the BioTrap column and carry away the retained analyte to the analytical column for chromatographic separation and detection. The composition of extraction mobile phase, flow rate, and the extraction time must be optimized to obtain maximum recovery of the analyte.

The applicability of on-line extraction with the C_{18} BioTrap 500 for in vitro drug metabolism studiesisdemonstratedhereusingtestosterone as the test analyte. Hydroxylation of testosterone to 6β-hydroxytestosterone is selectively catalyzed by the CYP 3A4 isozyme in liver microsomes. CYP 3A4 is one of the most important cytochrome P450 isoforms because of its involvement in the metabolism of a large number of drugs (2,3). When one isoform is responsible for the metabolism of a large number of endogenous compounds, there is a substantial possibility for drug-drug interactions to occur. Testosterone metabolism is generally used as a probe to detect inhibition or induction of CYP 3A4 by a test drug. Chemical inhibitors are used as a valuable tool in the identification of the specific isoform responsible for the metabolism of the test drug (4). Once the particular cytochrome P450 isoform is identified, drug-drug interactions can be predicted.

F1

Schematic diagram of the on-line extraction of analyte from biological samples and subsequent chromatographic separation. A more complete picture is on the BAS website, BioTrap section.



The present study describes the determination of testosterone and its hydroxy metabolite by direct injection of microsomal samples into the BioTrap column coupled to a C_{18} column using a 10-port injection valve. Although it is possible to use two BioTrap columns alternatively with the 10-port valve, only one BioTrap was used in this study.

Experimental Procedures

Reagents

Testosterone, 6β-hydroxytestosterone, and NADPH were purchased from Sigma Chemicals (St. Louis, MO). Sprague-Dawley male rat liver microsomes containing about 460 picomoles of cytochrome P450s per mg of protein were obtained from In Vitro Technologies (Baltimore, MD). All other chemicals used were of reagent grade or better. HPLC grade tetrahydrofuran (THF) was purchased from Aldrich (Milwaukee, WI).

Chromatography

The chromatographic system consisted of a BAS 200 with UV detector, PM-80 pump, CMA 200 autosampler, and a Pollen-8 on-line valve. A PM-80 pump was used to pump the extraction mobile phase through the BioTrap column. After a brief centrifugation step, 20 µL of the sample were directly injected onto the BioTrap column. Extraction mobile phase was 10 mM phosphate buffer (PB), pH 7.0 with 4% 2-propanol. The extraction time and the flow rate were optimized. A flow rate of 0.8 mL/min and an extraction time of 3 min were used for metabolism studies. Chromatographic separation was achieved isocratically with a mobile phase of 10 mM acetate buffer, pH 4.0/acetonitrile/THF (65/20/15, v/v) at 1.0 mL/min, on a 250x4.6 mm C_{18} column at 40º C. The complete chromatographic system coupled with the BioTrap C_{18} column and the 10-port valve arrangement is shown in ***F1***. Stock solutions of testosterone and hydroxytestosterone were prepared in methanol and diluted in phosphate buffer to obtain desired concentrations. Calibration standards were prepared by spiking 1 mg/mL microsomal protein solutions with testosterone and 6β-hydroxytestosterone. The standards were injected directly onto the BioTrap column.

Recovery of the BioTrap column was evaluated at several standard concentrations. The same testosterone and hydroxytestosterone standards (50, 75, 125 and 200 µM) were prepared in liver microsome solutions containing 1 mg/mL protein and in PB, pH 7.4. Standards containing microsomes were injected onto the BioTrap column. Aqueous standards were injected onto the analytical column directly after disconnecting the BioTrap and the 10-port valve. The peak areas were compared to determine the recovery. Recovery was evaluated as the percent ratio of peak areas of analyte in protein solution to analyte in buffer.

Microsomal Incubations

Rat liver microsomes (0.25 mg) were incubated with testosterone (range 10-500 µM) in a 0.5 mL polypropylene vial in 0.05 M potassium phosphate buffer (pH 7.4) containing 10 mM magnesium chloride at 37º C with magnetic stirring. The reaction was initiated by adding 25 µL of 10 mg/ml NADPH. The final volume of the sample was 250 µL. After a specified time interval, the reaction was stopped by adding 50 µL of 1 M perchloric acid. The samples were then centrifuged briefly. The supernatant was injected directly onto the BioTrap column.

The same incubations were carried out at different substrate concentrations ranging from 5 - 350 µM for 15 min to obtain Michaelis-Menten enzyme kinetics parameters for the hydroxylation of testosterone. The concentration-velocity data were fitted to the Lineweaver-Burk equation to obtain K_m and V_{max} values.

Results and Discussion

Identification of specific cytochrome P450 isoforms responsible for the metabolism of drugs using chemical inhibitors and inducers as investigational tools to predict drug-drug interactions is a common practice in drug metabolism studies (5,6). The suppression or induction of the specific cytochrome P450 isoform is investigated by measuring the activity of the inhibited or induced isozyme using a reaction specific to that isozyme. Testosterone metabolism to 6β-hydroxytestosterone is being used as a specific probe to detect both direct inhibition and activation of CYP 3A4 isozyme by a test compound. CYP 3A4 is responsible for the metabolism of a majority of clinically important drugs. The specificity of an inhibitor can be measured by measuring the hydroxylation activity of testosterone by CYP 3A4.

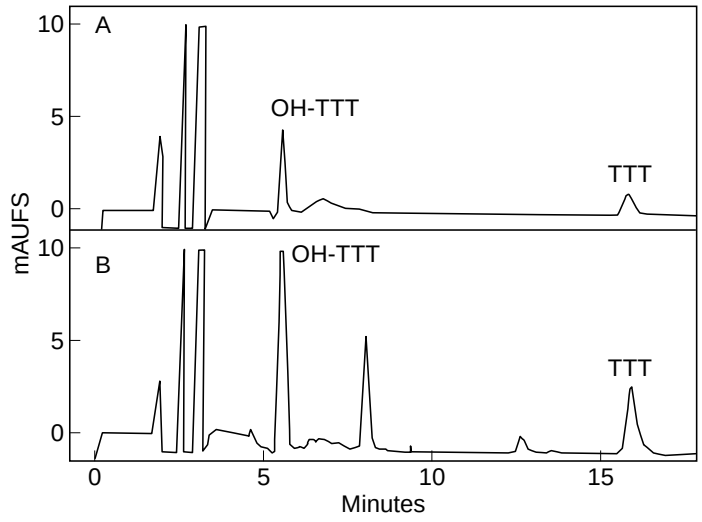
Several LC methods have been reported for the separation of testosterone and its hydroxylated metabo-

T1

Comparison of recovery (mean ± SD, for n=5) at different extraction times for testosterone (TTT) and hydroxytestosterone (OH-TTT)

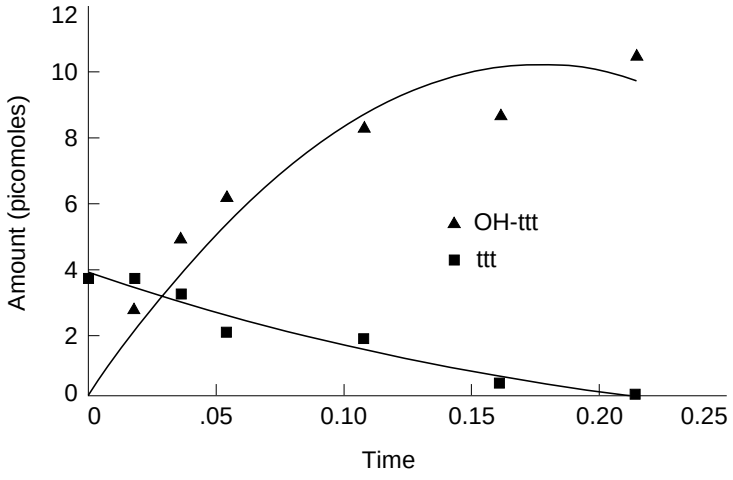
	Recovery (3 min)		Recovery (5 min)	
Concentration (μM)	**OH-TTT**	**TTT**	**OH-TTT**	**TTT**
50	100.7±0.2	100.0±3.7	85.2±0.9	86.0±3.5
75	97.8±1.5	106.1±4.9	84.7±2.8	98.5±2.9
125	99.5±1.1	97.8±1.4	81.7±3.7	102.3±0.5
200	96.6±0.7	94.1±2.5	92.6±1.2	101.1±0.3

F2



Typical chromatograms of A) standards of testosterone (TTT) and 6β-hydroxytestosterone (OH-TTT) in rat liver microsomes (1 mg/mL) and B) microsomal incubation sample after incubating 50 mM testosterone with 1 mg/mL rat liver microsomes in 0.05 M phosphate buffer, pH 7.4 with 10 mM $MgCl_2$ and 1.2 mM NADPH at 37° C for 15 min.

F3



Metabolic profiles of testosterone and 6β-hydroxytestosterone. Testosterone (76 μM) was incubated with 1 mg/mL rat liver microsomes in 0.05 M phosphate buffer, pH 7.4 with 10 mM $MgCl_2$ and 1.2 mM NADPH at 37° C for 5,10,15,30,45, and 60 min.

lites (7,8). Most of these methods involve gradient elution in order to achieve the separation of all the hydroxylated metabolites. Hydroxylated metabolites and testosterone are usually extracted from microsomes by liquid-liquid extraction into methylene chloride (8) or ethyl acetate (9). In this study we used an isocratic method to separate testosterone and the 6β-hydroxytestosterone. Although several hydroxy metabolites of testosterone are formed by the cytochrome P450 catalysis, only the formation of 6β-hydroxytestosterone was monitored, since 6β hydroxylation is the major route of metabolism (10). Microsomal samples were directly injected onto the BioTrap column for on-line extraction and then switched onto the analytical column for analysis. ***F2A*** shows a representative separation of testosterone and 6β-hydroxytestosterone standards in microsomes. With a mobile phase composition of 10 mM acetate buffer, pH 4.0/acetonitrile/THF (65/20/15, v/v), the retention times for 6β-hydroxytestosterone and testosterone were 5.3 and 15.7 min respectively. The retention times were very sensitive to the THF composition in the mobile phase. ***F2B*** shows a typical chromatogram of a microsomal incubation sample. A peak for an unidentified metabolite is present in the chromatogram in addition to 6β-hydroxytestosterone.

The intra-day coefficient of variation (CV) for the standards in microsomes was less than 5% (n=5) for both testosterone and the metabolite at 0.35 μM and less than 3% at 3.5 μM. Recovery of testosterone and 6β-hydroxytestosterone was evaluated at several different concentrations using extraction times of 3 and 5 min at 0.8-mL/min flow rate. The results tabulated in ***T1*** show that some loss of analyte occurs at longer extraction times for the more hydrophilic analyte whereas longer extraction time improves the recovery of the more hydrophobic component.

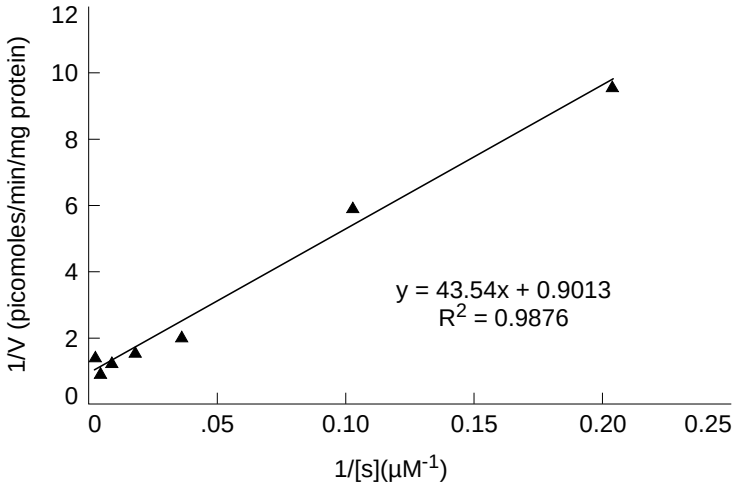
Formation of 6β-hydroxytestosterone and the disappearance of testosterone with time are shown in ***F3*** for a microsomal incubation containing 100 μM testosterone. Michaelis-Menten kinetics parameters, K_m and V_{max} were obtained by fitting substrate concentration, [S], and velocity, V, data into Lineweaver-Burke equation as follows:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

The Lineweaver-Burke plot ***(F4)*** for 6β-hydroxytestosterone formation was linear in the concentration range 5-200 μM. Deviation from linearity was observed at higher concentrations (~350 mM). The K_m for the hydroxylation reaction was 48.5 ± 3.7 μM and the V_{max} is 1.11 ± 0.08 nanomoles/min per mg protein (h=s).

F4

Lineweaver-Burke plot for the formation of 6β-hydroxytestosterone in the testosterone concentration range 5-350 µM.



Conclusion

Applicability of the BioTrap extraction column for fast, reproducible and sensitive assay of testosterone and its metabolite 6β-hydroxytestosterone in rat liver microsome samples has successfully been demonstrated. Microsomal samples were directly injected onto the analytical column using a BioTrap column to remove protein on-line. Extraction mobile phase composition, flow rate, and extraction time must be optimized to obtain high recovery of the analyte. This approach offers good precision for the assay and high recovery of analytes with no sample preparation step.

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