CDS Empore™

Extraction Disks

Method Summary

Imiquimod and Metabolites from Large Volumes of Urine

Summary

This method demonstrates the use of large diameter (47 mm) Empore Extraction Disks to purify and concentrate a novel immunomodulator drug, Imiquimod (Aldara™), and its metabolites from urine. In order to isolate sufficient mass of metabolites for analysis, some present in low concentrations, large volumes (10 to 100 mL) of animal and human urine were extracted. The eluates were analyzed by different chromatographic techniques (HPLC and TLC) to determine the nature and number of metabolites present. Samples were then analyzed by mass spectrometry, either LC/MS or GC/MS, to obtain supporting structural information about each metabolite.

Several types of biological samples were extracted (human, rat and rabbit urine and rat feces homogenate) onto sorbent-loaded disks [either C18 bonded silica, mixed phase cation bonded silica (C8/SCX), or SDB-XC poly(styrene divinylbenzene)]. The disks were washed of pigments and other impurities. Elution was performed with either methanol or successive organic solvents of increasing strength to selectively remove metabolites. For example, the metabolites of Imiquimod could be fractionated into acidic/neutral and basic portions when a mixed phase disk was used, and polar glucuronides could be separated from the parent aglycone using an SDB-XC disk.

Advantages of Disk Extraction

Empore Extraction Disks have been demonstrated to allow for:

- Rapid processing of large sample volumes
- Use of high flow rates
- Increased sample throughput
- Quantitative recoveries
- Clean extracts
- Increased sensitivity

Introduction

A commonly used technique to selectively isolate, concentrate and purify drugs and their metabolites from biological matrices prior to chromatographic or mass spectral analysis is solid phase extraction (SPE), using small disposable columns or cartridges. However, SPE cartridges present difficulties in processing large volumes of urine.

Disadvantages of SPE Cartridges:

- Narrow diameter (5-13 mm) and small volume reservoir (3-10 mL) limit sample throughput
- Must operate at low pressure to prevent channeling, thus increasing processing time
- Capacity can be insufficient for concentrating many metabolites present in low concentrations from a large sample volume
- Resulting eluates sometimes need further cleanup before GC/MS analysis to avoid source contamination

SPE in Disk Format

A technology has been developed by the 3M Company in which small (8 µm) chromatographic particles are enmeshed in a network of PTFE fibrils to form a strong, porous sheet or "particle-loaded membrane." Table 1 presents the general characteristics and flow properties of a typical SPE cartridge compared with a 47 mm Empore Extraction Disk. Since cartridges and disks have comparable packing mass (500 mg) but differ only in geometry, separations achieved on cartridges can be performed using disks containing similar sorbent. The use of a disk yields higher flow rates because of the lack of channeling, and demonstrates improved mass transfer kinetics as a result of the smaller particle size sorbent.

Table 1
Comparison of Typical SPE Cartridge and Empore Disk

Parameter	Cartridge	Empore Disk
Dimensions (height x diameter)	1.1 cm x 1.1 cm	0.05 cm x 4.7 cm
Cross-sectional area	0.95 cm^2	11.34 cm^2
Packing weight	500 mg	500 mg
Flow at 85 kPa*	30 mL/min	100 mL/min
Linear velocity†	0.525 cm/sec	0.150 cm/sec

*Typical
†at flow rate specified

Experimental

Empore Extraction Disks are 47 mm diameter, 0.5 mm thick and contain chromatographic particles (Table 2) immobilized within a stable, inert matrix of fibrillated polytetrafluoroethylene (PTFE). A patented processing method allows for high particle loading within the membrane (90% particles, 10% PTFE, w/w) with very uniform distribution *(Figure 1)*. The disks are used with standard 47 mm filtration glassware attached to a vacuum source.

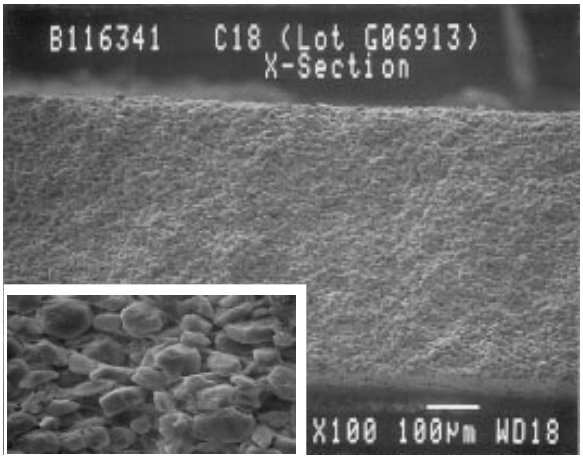

Figure 1. *Micrograph of a cross section of an Empore Extraction Disk containing C18 bonded silica particles enmeshed within PTFE fibrils.* ***Inset:*** *magnified 10x*

Flow Times

Flow times for processing volumes of human urine are summarized in Table 3. In general, flow was faster through a C18 disk (47 mm) when 3M Filter Aid 400 glass beads (10 g) were used as a prefilter medium to keep suspended solids from plugging the disk surface. The vacuum source was run as high as 15 in Hg with disks to yield faster flow times without affecting recovery (SPE columns were run at 5 in Hg according to manufacturer recommendations). Animal urine was more difficult to pass through the disks than human urine. Smaller volumes of 10-20 mL animal urine were used due to limited sample volume.

Table 3
Urine Flow Times

Urine Volume (mL)	SPE Cartridge 500mg/6mL	Empore C18 (without beads)	Empore C18 (with 10 g beads)
10	5 min	<1 min	<1 min
25	35 min	1 min	1 min
50	plugged	4 min	2 min
100	plugged	37 min	7 min

Recoveries

Radiolabled [^{14}C]-Imiquimod and metabolites in animal urine (obtained following oral administration), and spiked into human urine along with a known metabolite (radiolabled), were extracted with >95% recovery. Disk capacity was sufficient for isolating parent drug and many metabolites from urine as well as from animal feces homogenate.

Detection Techniques

Eluate fractions recovered from disks were analyzed by the following chromatographic techniques: HPLC, GC/MS, LC/MS, and TLC. Chromatograms from all detection methods showed clean baselines with reduced noise, allowing for increased sensitivity as shown in the following figures.

HPLC

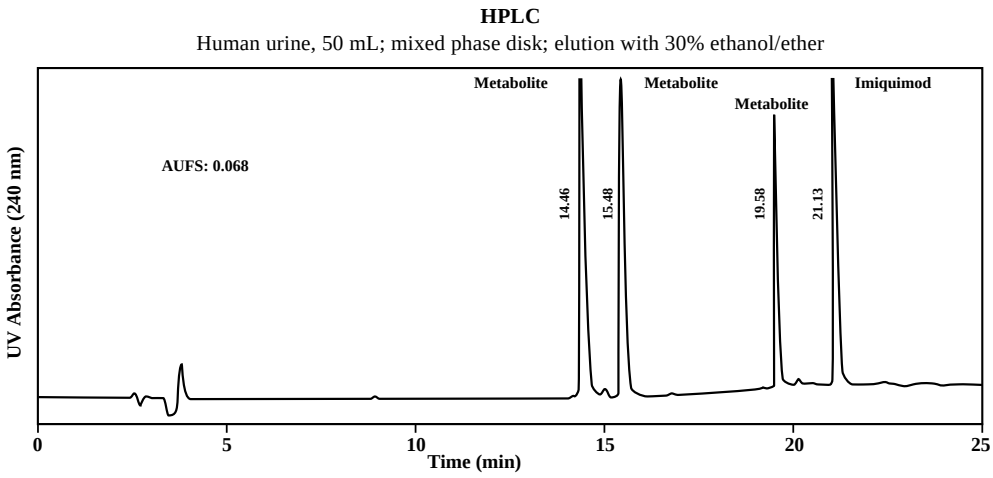

Figure 2. *Chromatogram of human urine (spiked at 1-2 µg/mL with parent drug and three known metabolites) after mixed phase disk extraction.*

GC/ MS

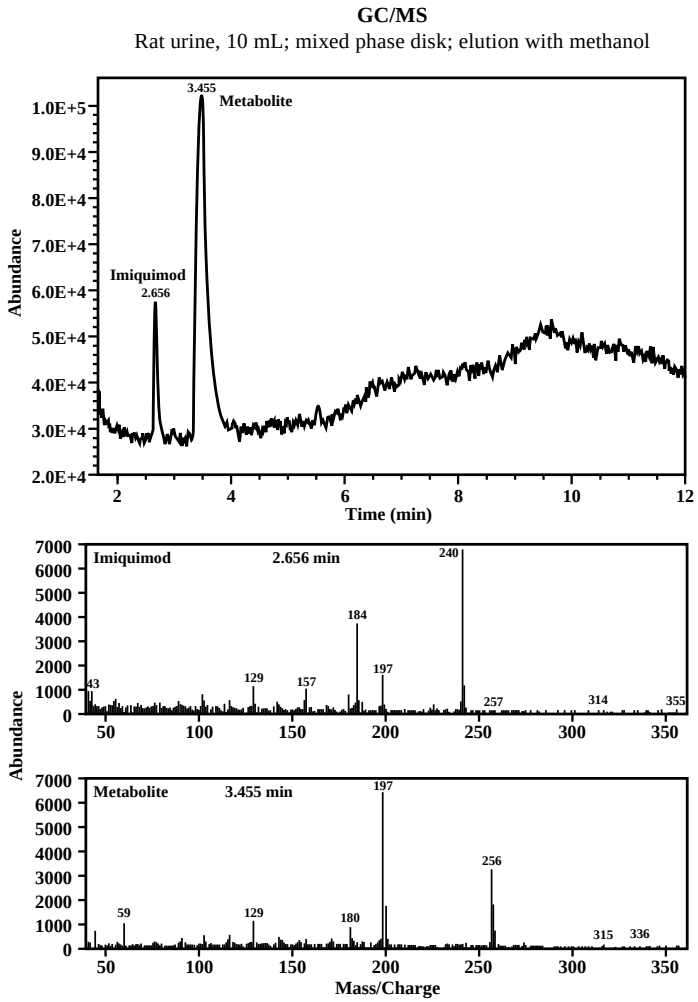

Figure 3. *Total ion chromatogram and mass spectra of rat urine (obtained following oral dosing of Imiquimod) after mixed phase disk extraction.*

LC/MS

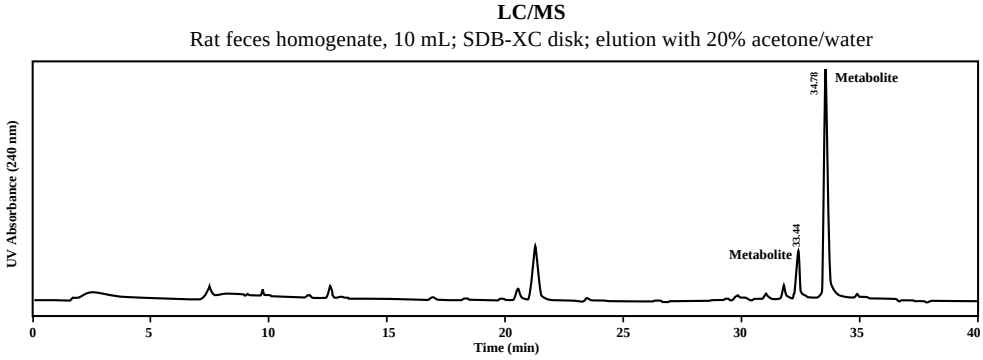

Figure 4. *Chromatogram of rat feces homogenate (obtained following oral dosing of Imiquimod) after SDB-XC disk extraction.*

Radiomonitored TLC

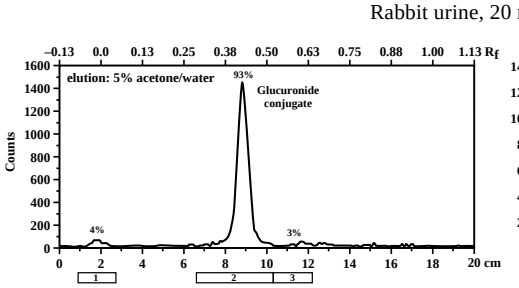

Figure 5. *TLC radiochromatogram of rabbit urine (obtained following oral dosing of [^{14}C]-Imiquimod) after SDB-XC extraction; glucuronide conjugate eluted.*

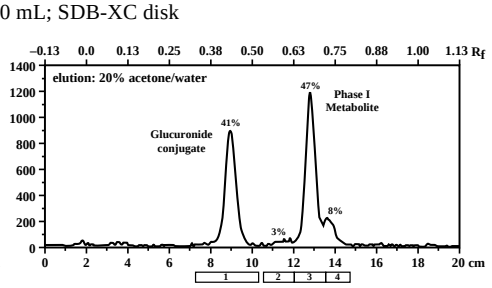

Figure 6. *TLC radiochromatogram of rabbit urine; phase I metabolite eluted with glucuronide conjugate.*

Objective of Study

Empore Extraction Disks have shown utility for drug sample preparation from serum and urine in many bioanalytical applications, as well as the ability to extract a wide range of pollutants from environmental water samples. The present study examined the potential of several types of Empore Extraction Disks (Table 2) to provide clean extracts for mass spectral analysis of metabolites of the drug Imiquimod and improve upon the disadvantages inherent with SPE cartridge techniques.

Table 2
Types of Sorbent-Loaded Disks Evaluated

Copolymerized C8 and SCX*	Poly(Styrene Divinylbenzene) SDB-XC	C18 Bonded Silica
Useful for fractionation of metabolites as acidic/neutral and basic	Useful for isolating polar glucuronide metabolites	Useful for retention of broad range of analytes with hydrophobic character
rat urine rat feces human urine	rabbit urine	human urine
Detection by LC/MS, GC/MS, HPLC or radiomonitored TLC		

*SCX = Strong cation exchange (benzene sulfonic acid)

Extraction Procedure

Prepare sample
- Adjust sample pH if necessary
- Add methanol to sample, 5% by volume *(fecal homogenate was first centrifuged and supernatant used)*

Condition disk
- Mount disk in glass filtration apparatus
- Add 5 mL methanol; apply vacuum; keep disk wet
- Add 5 mL of 20% methanol/water; apply vacuum; keep disk wet

Add sample
- Apply vacuum (10-20 in Hg) and allow sample to flow through
  Note: Recoveries are unaffected by sample flow rate

Wash disk

Mixed Phase (C8/SCX) or C18 Disk
- Wash with 5-10 mL of 20% methanol/water to remove urine pigments and glucuronides (retains phase I metabolites)

SDB-XC Disk
- Wash with 5-10 mL water (retains glucuronides and phase I metabolites)

Air dry disk
- Dry about 1-3 minutes at full vacuum

Elute
- Use successive 5-10 mL aliquots of organic solvents of increasing strength to collect metabolite fractions

Mixed Phase	SDB-XC	C18
30% ethanol/ether	5% acetone/water	methanol
methanol	20% acetone/water	

Conclusions

Empore Extraction Disks have been demonstrated to allow for:

- Rapid processing of large volumes (10 to 100 mL) of human and animal urine (and rat feces homogenate) by solid phase extraction
- Quantitative recoveries
- Sufficiently clean eluates for direct analysis by mass spectrometry
- Detection by a broad range of analytical techniques (HPLC, GC/MS, LC/MS and TLC)
- Reduced noise in chromatogram, allowing for increased sensitivity

Proper choice of sorbents and method optimization were shown to yield:

- Adequate removal of urinary pigments (C18 and mixed phase disk)
- Fractionation of metabolites as acidic/neutral and basic (mixed phase disk)
- Separation of polar glucuronides from parent aglycone (SDB-XC disk)

Reference

K. Ensing, J.P. Franke, A. Temmink, X. Chen and R.A. de Zeeuw, "Application of Empore C-8 Extraction Disks for Screening Urine in Systematic Toxicological Analysis," *Journal of Forensic Sciences*, 37(2), 460-466 (1992).

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