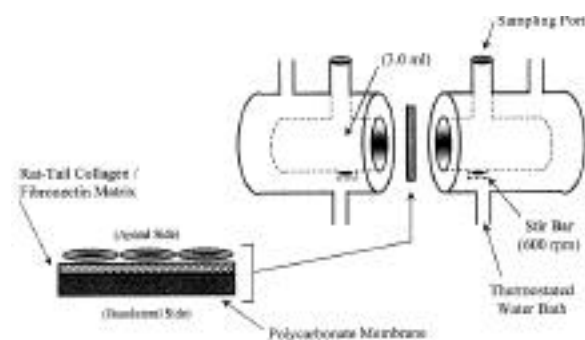


# Determination of substrate permeability in vitro by RP-HPLC and convective interaction media coupled disk HPLC

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**C**ONVECTIVE INTERACTION media (CIM) is a support matrix composed of cross-linked poly(glycidylmethacrylate-co-ethyleneglycoldimethacrylate) and is suitable for ion exchange, hydrophobic interactions, reversed-phase (RP), and affinity chromatography. A variety of functional groups coupled to CIM disks include quaternary amine, diethylamine, sulfonyl, octyl, and carboxymethyl (**IRIS Technologies**, Lawrence, KS). The disk dimensions, 16 mm (diam) × 3 mm, provide a high resolution of macromolecules and compounds within seconds and are compatible with conventional LC-HPLC equipment.

To compare the efficacy and benefits of CIM (SO<sub>3</sub>) (sulfonyl) (**IRIS Technologies**) with ODS C(18) (RP-HPLC), three compounds were investigated for limit of detection and peak resolution. The objective of this work was to measure the apparent permeability coefficients of three pharmacological compounds (acetaminophen, mol wt 151.2; diclofenac [Sigma Chemical Co., St. Louis, MO], mol wt 318.13; and testosterone, mol wt 288.4). The pharmacological properties of diclofenac, a phenylacetic acid derivative, acetaminophen, and testosterone have been well defined.<sup>1</sup> Both diclofenac and acetaminophen have analgesic and antipyretic properties. All of these compounds are mediated by passive diffusion through the cell monolayer. Apparent permeability coefficients provide approximate flux of transport via passive diffusion through biological barriers such as the intestinal mucosa, blood-brain endothelia, and placenta. The retention time(s) for acetaminophen, diclofenac, and testosterone were measured in seconds with CIM (SO<sub>3</sub>) HPLC. To identify a practical application of CIM-HPLC, the transport of these compounds across monolayers of the human trophoblast cell line BeWo was monitored to determine in vitro permeability. This study suggests that CIM (SO<sub>3</sub>) may be applicable to RP-HPLC and provides faster separations, high volumetric throughputs, and low backpressure.



**Figure 1** Side-Bi-Side diffusion apparatus. PBS was added in each of the donor and receiver chambers. The cells faced the donor chamber as the apical side, and the polycarbonate membrane faced the receiver chamber as the basolateral side. The monolayer cultures always faced the donor chamber of the diffusion system.

## BeWo cell culture

The BeWo cell line was obtained from Dr. Alan Schwartz at Washington University (St. Louis, MO). The cells were continuously cultured in Dulbecco's Modified Eagles Medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) containing 0.37% sodium bicarbonate and 1% antibiotic (10,000 U/mL penicillin and 10 mg/mL streptomycin). The cells were maintained in 150-cm flasks at pH 7.4 under 5% CO<sub>2</sub> and 95% humidity at 37 °C. The cells were collected by exposure to a trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.25% trypsin in phosphate-buffered saline [PBS]) and passed onto polycarbonate membranes (13 mm diam, 0.4 μm pore) coated with rat tail collagen in 100-mm Corning Costar (Cambridge, MA) culture dishes. Monolayer cell cultures were prepared with a typical seeding density of 100,000 cells/cm. The BeWo cell displays morphological properties common to the human placenta and provides an in vitro system to study transplacental transport.<sup>2</sup>

## Transmonolayer permeability

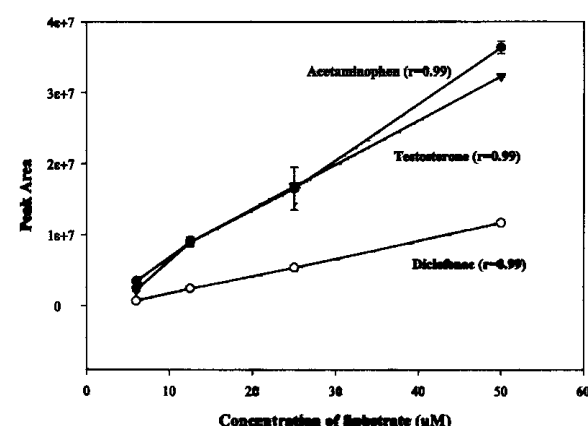
A horizontal Side-Bi-Side (**Crown Glass Co.**, Somerville, NJ) diffusion apparatus (*Figure 1*) was used to monitor the transport of each compound. The cells faced the donor chamber as the apical side, and the polycarbonate membrane faced the receiver chamber as the basolateral side. Polycarbonate filters treated with rat tail collagen but without cell monolayers were used as a control. The transport area was 0.636 cm in the diffusion system. The donor side final concentration of acetaminophen, diclofenac, and testosterone was 100 μM for all transmonolayer permeability studies. The contents of each chamber were continuously stirred at 600 revolutions/min with magnetic stir bars in the diffusion system.<sup>3</sup> An aliquot of 150 μL was taken at the scheduled time points (0–100 min), and the same volume (150 μL) of fresh PBS was replaced after each sample.

## Sample preparation

All aliquots taken from the receiver chamber were placed on the centrifuge for 3–5 min at 10,000 × g to remove cellular protein. All samples were injected directly onto either the ODS column or CIM (SO<sub>3</sub>) disk. To determine calibration plots, the samples were dissolved in mobile phase (65% acetonitrile, 0.6% dH<sub>2</sub>O, HOAc, and 0.25% triethylamine [TEA]) before injection onto the HPLC.

## Determination of apparent permeability coefficients

Analysis by RP-HPLC was determined with



**Figure 2** CIM (SO<sub>3</sub>) sulfonyl calibration plot of acetaminophen, diclofenac, and testosterone.

**Table 1**  
Retention time averages from three different samplings at a flow rate of 1 mL/min

Compound	CIM (SO <sub>3</sub> ) disk (min)	ODSHPLC (min)
Acetaminophen	0.45	1.2
Diclofenac	0.52	2.1
Testosterone	0.64	1.9

UV detection at 254 nm. Calibration plots and permeability experiments were determined with an ODS C(18), 3 μm (**Hypersil Life Sciences International**, London, England) 100 × 4.6 mm column (flow rate 1.0 mL/min) and a CIM (SO<sub>3</sub>) sulfonyl disk (flow rate 1.0 mL/min). Aliquots from permeability experiments and calibration plots of selected compounds were eluted with mobile phase (65% acetonitrile, 35% dH<sub>2</sub>O, 0.6% glacial acetic acid (HOAc), and 0.25% TEA) during isocratic conditions.

## Calculations and data analysis

Apparent permeability coefficients were estimated by the following equation:

$$P(\text{cm/s}) = X/(A \times t \times C_d)$$

Where P is the apparent permeability coefficient, x is the amount of substance (mol) in the receiver chamber at time t (sec), A is the diffusion area, and C<sub>d</sub> is the concentration of substance in the donor chamber (mol/cm).<sup>4</sup> C<sub>d</sub> remains >90% of the initial value over the time course of the experiments. The transport of the compounds studied, expressed as the flux [mol · cm<sup>-2</sup> · s<sup>-1</sup>], was determined as the mean ±SD from three to six different monolayers. The flux (mol/cm/sec) of a substance across the BeWo cell monolayers was calculated at the linearly regressed slope through linear data (sampling times 0–90 min).<sup>5</sup> The apparent permeability coefficient for the BeWo monolayers, P<sub>e</sub>, was calculated from the following relationship:

$$1/P_t = 1/P_e + 1/P_c$$

Table 2  
Apparent permeability coefficients for compounds determined by CIM (SO<sub>3</sub>) disk chromatography and RP-HPLC (ODS 3 μm)

Compound	CIM (SO <sub>3</sub> ) (cm/sec)	ODSRP-HPLC (cm/sec)	C14 label (cm/sec)
Acetaminophen	6.11 ± 1.2 × 10 (4)	4.63 ± 1.1 × 10 (4)	5.7 × 10 (5)
Diclofenac	2.16 ± 1.5 × 10 (4)	1.7 ± 0.1 × 10 (4)	—
Testosterone	5.16 ± 0.18 × 10 (4)	2.57 ± 0.28 × 10 (4)	2.9 ± 1.0 × 10 (4)

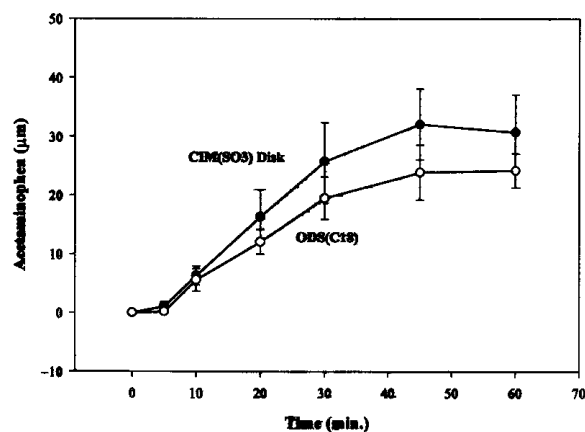


Figure 3 In vitro acetaminophen concentration (μM) measured with ODS-HPLC and CIM(SO<sub>3</sub>) disk chromatography.

where  $P_t$  is the apparent permeability coefficient for the collagen-coated polycarbonate membranes in the presence of BeWo cell monolayers, and  $P_c$  is the apparent coefficient for collagen-coated polycarbonate membranes alone.<sup>6</sup>

## Results

### Calibration plots of acetaminophen, diclofenac, and testosterone

To calculate the concentration of each compound in the Side-Bi-Side receiver chamber, calibration plots were generated. In Figure 2, each selected compound was prepared in mobile phase and was injected into the HPLC system. With UV absorbance (254 nm), detection of the lowest concentration (6 μM) for each compound was comparable in both methods. The retention time of each compound shown in Table 1 is the average for each chromatogram. In these experiments with CIM (SO<sub>3</sub>) disk chromatography, the retention times for acetaminophen, diclofenac, and testosterone were reduced by 50% compared to the ODS column.

### Apparent permeability coefficient

To determine the apparent permeability coefficients, the flux of each compound across the BeWo monolayers was monitored for 90 min in the diffusion system. Table 2 is a summary of permeability coefficients for acetaminophen, diclofenac, and testosterone given in cm/sec units. The data in Table 1 indicate similar apparent permeability coefficient(s) for acetaminophen, testosterone, and diclofenac with CIM chromatography and ODS (C18) RP-HPLC. In all permeability experiments (Figures 3–5), an observed saturation in permeability below the donor concentration ( $C_d = 100$  μM) was observed with acetaminophen, diclofenac, and testosterone.

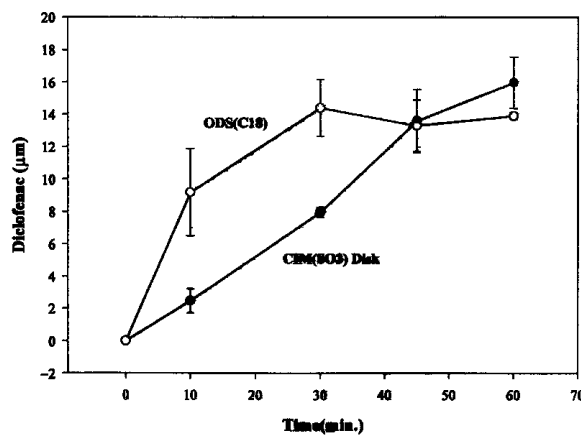


Figure 4 In vitro diclofenac concentration (μM) measured with ODS-HPLC and CIM (SO<sub>3</sub>) disk chromatography.

## Discussion

The results observed in these experiments include a practical application for CIM chromatography. CIM disks are designed for fast analysis and identification of target biomolecules. Previous reports included separation and purification of peptides, proteins, enzymes, or antibodies with various functional groups supported by CIM. A practical application supported by data in this report suggests sulfonyl CIM disk chromatography for the separation of low-molecular-weight compounds (acetaminophen, mol wt 151.2; diclofenac, mol wt 318.1; and testosterone, mol wt 288.4). The results observed using the CIM-C8 (octyl) and CIM-QA (quaternary amine) also provided a practical method for the separation of these compounds.

The apparent permeability coefficients derived in these experiments were similar to radiolabeled (C14) acetaminophen and testosterone. Permeability coefficients calculated from in vitro experiments with BeWo cell transmonolayer were  $5.7 \times 10 (5)$  cm/sec and  $1.23 \pm 0.33 \times 10 (4)$  cm/sec for acetaminophen and testosterone, respectively. In comparing the CIM (SO<sub>3</sub>) and ODS (C18) disks, acetaminophen permeability was  $6.11 \pm 1.2 \times 10 (4)$  cm/sec and  $4.63 \pm 1.1 \times 10 (4)$  cm/sec, which may reflect the ionic charge interaction of acetaminophen with transmembrane matrices. In previous experiments, apparent permeability coefficients of C14 labeled testosterone were  $2.9 \pm 1.0 \times 10 (4)$  and  $1.23 \pm 0.33 \times 10 (4)$  cm/sec. The observed difference in testosterone permeability,  $5.18 \pm 0.18 \times 10 (4)$  cm/sec and  $2.57 \pm 0.28 \times 10 (4)$  cm/sec with CIM (SO<sub>3</sub>) and ODS (C18), are in agreement with the radiolabeled compound (see Table 3).

## Conclusion

CIM (SO<sub>3</sub>) chromatography provided faster separation of molecules with resolution comparable to the ODS (C18) column. Convective interaction media are chromatographic and bioconversion supports based on a highly cross-linked porous polymer, offering high chemical stability and high flow characteristics. They are available in different forms and chemistries, making them suitable for ion exchange, hydrophobic interaction, reversed-phase, and preparative scale. The supports combine the advantages of chromatographic columns packed with conventional porous particles in terms of separation power, capacity, and sample distribution and those of membrane technology with regard to convective

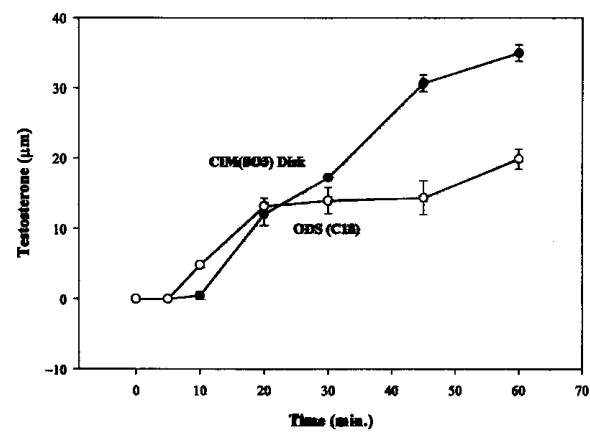


Figure 5 In vitro testosterone concentration (μM) measured with ODS-HPLC and CIM (SO<sub>3</sub>) disk chromatography.

Table 3

### Determination of apparent permeability coefficient. Concentration (μM) of acetaminophen, diclofenac, and testosterone in the receiver chamber

Time (min)	Acetaminophen (μM)	
	ODS	CIM(SO <sub>3</sub> )
5	0.17 ± 0.08	1.1 ± 0.705
10	5.57 ± 1.9	6.35 ± 1.60
20	12.1 ± 2.1	16.4 ± 4.6
30	19.55 ± 3.6	25.8 ± 6.6
45	23.9 ± 4.7	32.1 ± 6.05
60	24.2 ± 2.9	30.7 ± 6.4
Time (min)	Diclofenac (μM)	
	ODS	CIM (SO <sub>3</sub> )
10	9.2 ± 2.7	2.47 ± 0.75
30	14.4 ± 1.7	7.97 ± 0.3
45	13.3 ± 1.6	13.6 ± 1.96
60	13.9 ± 0.21	15.96 ± 1.6
Time (min)	Testosterone (μM)	
	ODS	CIM (SO <sub>3</sub> )
5	0.0	0.0
10	4.8 ± 0.4	0.45 ± 0.2
20	13.2 ± 1.1	12.1 ± 1.6
30	14.0 ± 1.86	17.3 ± 0.42
45	14.4 ± 2.4	30.7 ± 1.2
60	19.9 ± 1.4	35.02 ± 1.2

mass transport. This results in much faster separations, high volumetric throughputs, and low backpressure. As an alternative method to RP-HPLC, use of CIM chromatography with nonradioactive compounds may be useful in the quantitative analysis of both hydrophilic and hydrophobic compounds. In comparison with the hydrophilic compounds, diclofenac and acetaminophen, analysis of testosterone was measured with CIM (SO<sub>3</sub>) using isocratic elution. These results suggest that compounds of low molecular weight are also detected by methods developed for hydrophobic macromolecules.

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