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Short communication

Development and characterization of immobilized human organic anion transporter-based liquid chromatographic stationary phase: hOAT1 and hOAT2

T. Kimura [a], J. Perry [b], N. Anzai [c], J.B. Pritchard [b], R. Moaddel [a,*]

[a] *Gerontology Research Center, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA*
[b] *National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA*
[c] *Department of Pharmacology and Toxicology, Kyorin University School of Medicine, 6-20-02 Shinkawa, Mitaki-Shi, Tokyo 181-8611, Japan*

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Abstract

This paper reports the development of liquid chromatographic columns containing immobilized organic anion transporters (hOAT1 and hOAT2). Cellular membrane fragments from MDCK cells expressing hOAT1 and S2 cells expressing hOAT2 were immobilized on the surface of the immobilized artificial membrane (IAM) liquid chromatographic stationary phase. The resulting stationary phases were characterized by frontal affinity chromatography, using the marker ligand [^{3}H]-adefovir for the hOAT1 and [^{14}C]-*p*-aminohippurate for the hOAT2 in the presence of multiple displacers. The determined binding affinities (K_d) for eight OAT1 ligands and eight OAT2 ligands were correlated with literature values and a statistically significant correlation was obtained for both the hOAT1 and hOAT2 columns: $r^2 = 0.688$ ($p < 0.05$) and $r^2 = 0.9967$ ($p < 0.0001$), respectively. The results indicate that the OAT1 and OAT2 have been successfully immobilized with retention of their binding activity. The use of these columns to identify ligands to the respective transporters will be presented.
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Keywords: hOAT1; hOAT2; Drug transporters; Affinity chromatography

1. Introduction

Transport proteins are found in the liver, kidney and intestine and play an essential role in the absorption and excretion of endogenous and exogenous compounds. These proteins have become a target in drug development and drug discovery [1]. The solute carrier (SLC) family contains 298 members in humans of which the majority are highly specific [2]. However, some of the families are polyspecific, including SLC 21 and SLC 22.

The SLC 22 superfamily has 12 members in humans, these include organic cation transporters, carnitine transporters and several organic anion transporters (OATs). Several families of multispecific organic anion transporters have recently been identified by molecular cloning, including hOAT1 [3], hOAT2 [4], hOAT3 [5] and hOAT4 [6]. Among the OAT families, hOAT1 (*SLC22A6*), hOAT3 (*SLC22A8*) and hOAT4 (*SLC22A11*) are predominantly expressed in the human kidney, whereas hOAT2 (*SLC22A7)* is highly expressed in the human liver and weakly in the human kidney [7,8].

OATs play an important role in the distribution and excretion of endogenous (cyclic nucleotides and dicarboxylates) and exogenous (mycotoxins, sulfate, glucoronide and glycine conjugates) drugs, as they are typically found at boundary epithelia [1,9,10]. They are predominantly anion exchangers and in the kidney, for example, are functionally coupled to sodium driven mono- and di-carboxylate transporters [1]. It has been reported that in some cases interactions with the OATs may result in pharmacokinetic drug–drug interactions or nephrotoxicity [11,12].

While OATs are multispecific, the subtypes appear to have different selectivities. The hOAT1 contributes to uptake of a

* Corresponding author at: Bioanalytical and Drug Discovery Unit, National Institute on Aging, National Institutes of Health, Gerontology Research Center, 5600 Nathan Shock Drive, Baltimore, MD 21224-6825, USA.
Tel.: +1 410 558 8294; fax: +1 410 558 8409.
E-mail address: moaddelru@grc.nia.nih.gov (R. Moaddel).

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range of small organic anions across the basolateral membrane of the renal proximal tubule and drives their urinary elimination. Several recent studies indicated that the OAT1 is involved in the tubular secretion of many important therapeutics such as β-lactam antibiotics, nonsteroidal anti-inflammatory drugs and antiviral nucleotide analogs [12–14].

Identified hOAT2 substrates include methotrexate, prostaglandin E2, cAMP, azidodeoxythymidine (AZT) and tetracycline [15–17]. However, there is still limited data concerning the substrate selectivity and transport mechanism of hOAT2.

The measurement of the binding affinities of OAT substrates or inhibitors is a key step in the study of transport mechanism and in the identification of drug–transporter interactions. Currently, OAT affinities are measured by cellular uptake studies, which are used to determine IC_{50} and K_is values [11]. Although this method provides reliable results, it is time consuming.

This laboratory previously developed an alternative method for the study of binding interactions between compounds and receptors or drug transporters [18,19]. This approach is based upon liquid chromatography utilizing stationary phases containing immobilized membranes from cells expressing the target protein. For example, binding to the human organic cation transporter (hOCT1) has been studied using a column created using membranes from a cell line expressing hOCT1. The membranes were immobilized on an immobilized artificial membrane (IAM) liquid chromatographic stationary phase. The OCT1-IAM stationary phases were used in frontal affinity chromatography studies to determine the binding affinities (K_d values) of OCT substrates and inhibitors and were able to identify competitive and enantioselective interactions between ligands and the OCT1 transporter.

In the current study, this experimental approach has been extended to the development of hOAT1-IAM and hOAT2-IAM stationary phases. The columns were prepared using MDCK (canine kidney) cells stably transfected with hOAT1 [20] and S2 (mouse kidney) cells stably transfected with hOAT2 [21]. Columns were prepared from both stationary phases and tested to determine the binding activity and specificity of the immobilized membranes containing hOAT1 and hOAT2. The columns were characterized using frontal displacement chromatography with [^{3}H]-adefovir for hOAT1 [12,11] and [^{14}C]-PAH for hOAT2 [4] as the marker ligand and known hOAT1 and hOAT2 ligands as the displacers. The results demonstrate that the hOAT1 and hOAT2 membranes were successfully immobilized on the IAM stationary phase and they retained the ability to specifically bind known ligands from which K_d values could be determined.

2. Experimental

2.1. Materials

Glutarate, *p*-aminohippurate (PAH), probenecid, indomethacin, 3′-azido-3′-deoxythymidine (AZT), ketoprofen, methotrexate (MTX), diclofenac, mefenamic acid, cholate, leupeptin, phenyl methyl sulfonyl fluoride (PMSF), EDTA, Trizma, glycerol, pepstatin, sodium chloride, $MgSO_4$, $CaCl_2$, and 6-carboxyfluorescein were purchased from Sigma (St. Louis, MO, USA). Ammonium acetate and 0.1 M ammonium hydroxide solution were purchased from Fisher Scientific (Pittsburgh, PA, USA). 9-(2-Phosphonylmethoxyethyl) adenine ([2,8-^{3}H] adefovir; 10 Ci/mmol) and adefovir were purchased from Moravek Biochemicals (Brea, CA, USA). [^{14}C]-PAH was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Minimum essential medium with Earle's salts and L-glutamine (MEM), fetal bovine serum (FBS) and geneticin were purchased from Gibco (Carlsbad, CA, USA). RITC 80-7 medium was kindly provided by Dr. Naohiko Anzai (Kyorin University, Tokyo, Japan). Immobilized artificial membrane stationary phase (IAM-PC, 12 μm particle size, 300 Å pore size) was purchased from Regis Technologies Inc. (Morton Grove, IL, USA). HR 5/2 glass columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

2.2. Methods

2.2.1. Cell lines

The hOAT1 membranes were obtained from a previously described hOAT1-MDCK cell line were maintained as previously described [20]. In brief, cells were maintained in EMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1 mM Na-pyruvate and 200 μg/ml geneticin (G418) in a humidified incubator at 37 °C with 95% O_2 and 5% CO_2. Cells were split 1:10 every 7 days and were subcultured into new flask.

The hOAT2 membranes were obtained from S2 cells stably expressing hOAT2 and established as described previously [22]. In brief, cells were grown in RITC 80-7 medium containing 5% fetal bovine serum, 10 μg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor and 400 μg/ml geneticin in a humidified incubator at 33 °C and 5% CO_2 atmosphere. The cells were subcultured in a medium containing 0.05% trypsin–EDTA solution.

2.2.2. Preparation of the hOAT1-IAM and hOAT2-IAM columns

The hOAT1-MDCK cells and hOAT2-S2 cells (100×10^6 cells) were placed in 10 ml of homogenization buffer (Tris–HCl [50 mM, pH 7.4] containing 50 mM NaCl, 8 μM leupeptin, 10 μM PMSF and 8 μM pepstatin). The suspension was homogenized for 3×10 s at the setting of 12.5 on a Model PT-2100 homogenizer (Kinematica AG, Luzern, Switzerland). The homogenate was centrifuged at 27,000 rpm for 35 min at 4 °C and the resulting pellet containing the cellular membranes was collected and resuspended in 10 ml of solubilization buffer (Tris–HCl [50 mM, pH 7.4], containing 2% cholate, 50 mM NaCl, 10 μM PMSF, 8 μM pepstatin A, 10 μM leupeptin, 10 μM aprotinin and 10% glycerol). The resulting mixture was rotated at 150 rpm using an orbit shaker (Lab-line Model 3520, Melrose Park, IL, USA) for 18 h at 4 °C and then it was centrifuged at 20,000 × rpm for 22 min. Subsequently, 160 mg of the IAM stationary phase was suspended in the supernatant containing hOAT1-cholate solution, and the resulting mixture was rotated at room temperature for 3 h at 150 rpm using an orbit shaker

and then dialyzed against dialysis buffer (Tris–HCl [50 mM, pH 7.4] containing 150 mM NaCl, 1 mM EDTA and 1 mM benzamidine) for 2 days. The resulting mixture was centrifuged for 3 min at 4 °C at 700 × *g* and the supernatant was discarded. The pellet (hOAT1-IAM or hOAT2-IAM) was washed with Tris–HCl [10 mM, pH 7.4] and centrifuged. This process was repeated until the supernatant was clear.

2.2.3. Frontal chromatography with radiolabeled markers

2.2.3.1. Chromatographic system. The hOAT1-IAM and hOAT2-IAM columns were packed into a HR 5/2 glass column to yield a 150 mm × 5 mm (I.D.) chromatographic bed. These columns were connected to a LC-10AD isocratic HPLC pump (Shimadzu, Columbia, MD, USA). The mobile phase consisted of Tris–HCl [10 mM, pH 7.4] containing 1 mM $CaCl_2$ and 0.5 mM $MgSO_4$ delivered at 0.2 ml/min at room temperature. Detections of the [^{3}H]-adefovir for OAT1 and [^{14}C]-PAH for OAT2 were accomplished using an on-line scintillation detector (IN/US system, β-ram Model 3, Tampa, FL, USA) with a dwell time of 2 s using Laura lite 3.

2.2.3.2. Chromatographic studies. The marker ligands for the frontal chromatographic studies on hOAT1 and hOAT2 were [^{3}H]-adefovir (0.3 nM) and [^{14}C]-PAH (80 nM), respectively. In the chromatographic studies, a 50 ml sample Superloop (Amersham Pharmacia Biotech) was used to apply the marker ligand and a series of displacer ligands: glutarate (1, 5, 10, 20 and 50 μM), PAH (1, 10, 100, 200 and 300 μM), probenecid (1, 10, 100, 200 and 300 μM), adefovir (0.5, 1, 10, 20 and 30 μM), mefenamic acid (0.1, 0.2, 0.3, 0.4 and 0.5 μM), diclofenac (0.5, 4, 5, 6 and 10 μM), indomethacin (1, 2, 4, 10 and 20 μM) and 6-carboxyfluorescein (1, 10, 50, 100 and 300 μM) for hOAT1; AZT (0.1, 5, 10, 100 and 200 μM), *p*-aminohippurate (5, 10, 100, 200 and 400 μM), probenecid (50, 100, 200, 250 and 300 μM), indomethacin (10, 20, 100, 200 and 250 μM), diclofenac (5, 10, 30, 50, 100 and 200 μM), ketoprofen (200, 300, 400, 500 and 600 μM), methotrexate (MTX) (10, 20, 30 40 and 50 μM) and mefenamic acid (10, 20, 30, 40 and 50 μM) for hOAT2.

2.3. Data analysis

The dissociation constants for the marker (K_{dM}) and displacer ligands (K_d) were calculated using a previously described linear and non-linear approach [18,19]. Both approaches resulted in similar K_ds, for example, methotrexate was calculated to be 9.05 and 8.90 μM, respectively. As the methods only have minor differences between them we will only report those obtained by the non-linear approach. In brief, the dissociation constants of displacer ligand (K_d) can be calculated using Eq. (1), assuming that the [M] ≪ K_d of marker, so that [M]/K_{dM} ≪ 1:

$$[D]\,(V - V_{min}) = P[D](K_d + [D])^{-1} \quad (1)$$

where [*D*] is the concentration of displacer ligand, *V* the retention volume of displacer ligand, V_{min} the retention volume of displacer ligand when the specific interaction is completely suppressed and *P* is the product of the B_{max} (the number of active binding sites) and (K_d/K_{dM}). From the plot of [*D*] ($V - V_{min}$) versus [*D*], dissociation constant values (K_d), for displacer ligand can be obtained. The data was analyzed by non-linear regression with a rectangular hyperbolic curve using Prism 4 software (Graph Pad Software Inc., San Diego, CA, USA) running on a personal computer.

3. Results and discussion

3.1. K_d values from hOAT1- and hOAT2-IAM columns

In frontal chromatography, a marker ligand that is specific for the immobilized target is placed in the mobile phase and passed through the column, and elution profiles containing front and plateau regions are then obtained [23]. The frontal region is the relatively flat initial portion of the chromatographic trace that reflects the binding of the marker to the target up to the saturation, which is represented by a vertical breakthrough and attainment of a plateau region. In this study the immobilized target was hOAT1 and hOAT2 and the specific marker was 0.3 nM adefovir and 80 nM PAH, respectively. When [^{3}H]-adefovir was chromatographed alone, the midpoint of the breakthrough curve was observed at 7 min, representing a breakthrough volume of 1.4 ml, while for [^{14}C]-PAH the midpoint of the breakthrough curve was observed at 4.85 min, representing a breakthrough volume of 0.97 ml.

In a competitive displacement study, increasing concentrations of a competing ligand are added to the mobile phase, and the displacement of the breakthrough curve are determined and used to calculate the K_d of the competing ligand using Eq. (1). For the hOAT1, the addition of 20 and 30 μM adefovir to the running buffer produced concentration-dependent decreases in marker ligand retentions (Fig. 1). The results indicate that the binding activity of the immobilized hOAT1 could be probed using affinity displacement chromatography.

The dissociation constant (K_d) of PAH, glutarate, probenecid, 6-carboxyfluorescein, mefenamic acid, diclofenac, indomethacin and adefovir were determined for the hOAT1-IAM column using frontal displacement chromatography with

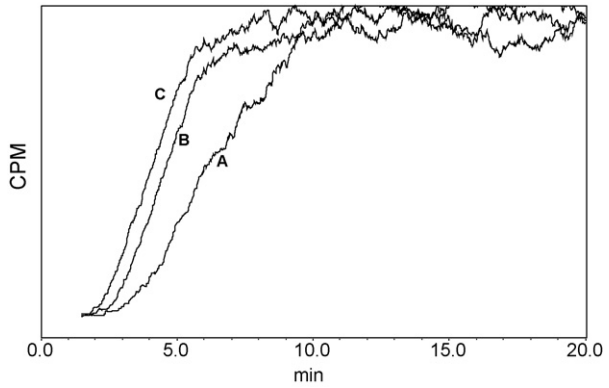

Fig. 1. The effect of the addition of increasing concentrations of adefovir on the chromatographic retention of 300 pM [^{3}H]-adefovir on the hOAT1-IAM column from no adefovir in the mobile phase (A) to 20 μM adefovir in the mobile phase (B) to 30 μM adefovir in the mobile phase (C).

Table 1
Binding affinities (K_d) values calculated using frontal affinity chromatography on an immobilized hOAT1 column, compared to IC_{50} values calculated using cellular uptake studies [11,24–26]

Compound	K_d (μM)	IC_{50} (μM)
p-Aminohippurate	48.5 ± 15.4	8.8 [26]
Glutarate	38.3 ± 5.97	4.9 [26]
Probenecid	45.7 ± 15.3	6.3 [26] 12.3 [24,25]
6-Carboxyfluorescein	39.9 ± 17.8	Not reported
Adefovir	64.0 ± 5.14	28 [11]
Mefenamic acid	0.14 ± 0.05	0.83 [25]
Diclofenac	6.07 ± 3.02	3.83 [25]
Indomethacin	4.40 ± 1.70	0.14 [25]

[^{3}H]-adefovir as the marker ligand (Table 1). The competitive frontal displacement of [^{3}H]-adefovir was seen with all the ligands tested. As shown in Table 1, the K_d values were compared with IC_{50} values, obtained from cellular uptake studies, with the exception of 6-carboxyfluorescein. The K_d values calculated using the chromatographic method correlated with those determined from the membrane binding techniques with an r^2 value of 0.688 ($p < 0.05$), $n = 7$, using a linear regression analysis on Graph Pad Prism version 4.0.

The frontal displacement chromatography method was also applied to the hOAT2-IAM column. The K_d of AZT, indomethacin, mefenamic acid, diclofenac, probenecid, adefovir and PAH were determined on the hOAT2-IAM column (Table 2). The K_d values of the test compounds with the exception of AZT, MTX and PAH were compared to previously reported IC_{50} values. Similar to hOAT1, the chromatographically determined dissociation constants correlated with previously reported IC_{50} values using a linear regression analysis on Graph Pad ver 4.0 with an r^2 value of 0.9967 ($p < 0.0001$), $n = 5$. Thus, the results indicate that the hOAT2 transporter retained its activity on the hOAT2-IAM column.

3.2. hOAT1- and hOAT2-IAM column selectivity

The frontal displacement chromatography method was also applied to confirm the selectivity of the columns. Adefovir, for example, was also used as a displacer for hOAT2. However, unlike hOAT1 which saw a significant displacement with 30 μM adefovir, the addition of 100 μM adefovir to the mobile phase produced no displacement of PAH on the hOAT2 column (data not shown). This result indicates that, under the experimental conditions used in this study, adefovir did not compete with PAH for binding to the immobilized hOAT2 transporter. Similar results were observed with MTX. The addition of MTX to the running buffer on the hOAT2 produced a concentration-dependent decrease in the retention of [^{14}C]-PAH for the hOAT2 column, but had no effect on the retention of [^{3}H]-adefovir on hOAT1 column (Fig. 2).

Table 2
Binding affinities (K_d) values calculated using frontal affinity chromatography on an immobilized hOAT2 column, compared to IC_{50} values calculated using cellular uptake studies [25,27]

Compound	K_d (μM)	IC_{50} (μM)
AZT	81.0 ± 12.2	Not reported
Indomethacin	49.5 ± 5.95	64.1 [25]
Diclofenac	18.7 ± 12.3	14.3 [25]
Mefenamic acid	20.6 ± 0.85	21.7 [25]
Probenecid	409.5 ± 113.0	668 [24]
p-Aminohippurate	108.9 ± 34.0	Not reported
Methotrexate	8.9 ± 0.93	Not reported
Ketoprofen	272.8 ± 77.9	400 [25]

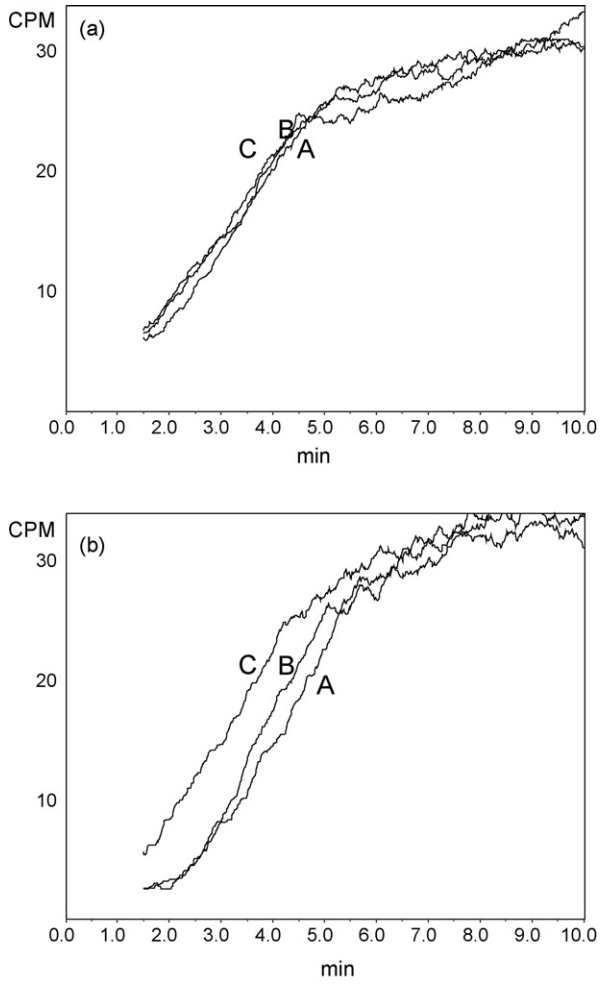

Fig. 2. The effect of the addition of increasing concentrations of MTX on the chromatographic retention 300 pM [^{3}H]-adefovir on the hOAT1-IAM column (a) and 80 nM [^{14}C]-PAH hOAT2-IAM column (b) from 10 μM MTX in the mobile phase (A) to 20 μM MTX in the mobile phase (B) to 30 μM MTX in the mobile phase (C).

4. Conclusions

The data from this study indicate that membranes from the hOAT1-MDCK and hOAT2-S2 cell lines have been successfully immobilized onto the IAM stationary phase, creating hOAT1-IAM and hOAT2-IAM stationary phases. And that these columns could be useful in the investigation of drug–transporter interactions. The data also demonstrate that the binding affinities calculated by frontal displacement chromatography correlated with IC_{50} values obtained using cellular uptake studies, sug-

gesting that this method can be used in place of current cellular uptake studies, which are time consuming and laborious. In addition, this technique can be used for a preliminary screen for drug candidates using the differences of their binding affinities, and be also used for finding the mechanism of action of the transporter using in vivo substrate as a displacer.

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