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Development and characterization of an immobilized human organic cation transporter based liquid chromatographic stationary phase

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Abstract

Membranes from a stably transfected cell line that expresses the human organic cation 1 transporter (hOCT1) have been immobilized on the immobilized artificial membrane (IAM) liquid chromatographic stationary phase to form the hOCT1(+)-IAM stationary phase. Membranes from the parent cell line that does not express the hOCT1 were also immobilized to create the hOCT1(−)-IAM stationary phase. Columns were created using both stationary phases, and frontal displacement chromatography experiments were conducted using [^{3}H]-methyl phenyl pyridinium ([^{3}H]-MPP^+) as the marker ligand and MPP^+, verapamil, quinidine, quinine, nicotine, dopamine and vinblastin as the displacers. The K_d values calculated from the chromatographic studies correlated with previously reported K_i values ($r^2 = 0.9987$; $p < 0.001$). The data indicate that the hOCT1(+)-IAM column can be used for the on-line determination of binding affinities to the hOCT1 and that these affinities are comparable to those obtained using cellular uptake studies. In addition, the chromatographic method was able to identify a previously undetected high affinity binding site for MPP^+ and to determine that hOCT1 bound (*R*)-verapamil to a greater extent than (*S*)-verapamil.
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1. Introduction

Transport proteins are found in the liver, kidney and intestines and play an essential role in the metabolism and excretion of endogenous and exogenous compounds [1–4]. The solute carrier (SLC) transporters have 255 members in humans, the majority are highly specific transporters, however, some of the superfamilies are polyspecific.

Two polyspecific transporters superfamilies that are of particular interest in the drug development process are the SLC21 and SLC22 superfamilies. The SLC21 superfamily (organic anion transporter) is composed of nine members, which are involved in the transport of large anionic, amphipathic compounds. The SLC22 superfamily (major facilitator superfamily) [2] has 12 members in humans and rats including the organic cation transporters OCT1, OCT2 and OCT3, the carnitine transporter, and several organic anion transporters. The rOCT1 has been isolated from rats and shown to consist of 556 amino acid residues with 12 transmembrane domains and three glycosylation sites on the extracellular loop between the first and second transmembrane domain [3,4].

OCTs are believed to mediate the bidirectional transport of small organic cations (50–350 amu) such as tetraethyl ammonium (TEA) and 1-methyl-4-phenyl pyridinium (MPP^+)

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[1,3]. Other compounds have been shown to bind to the human OCT1 (hOCT1) without being transported, and have been shown to act as transport inhibitors. These compounds include verapamil, quinidine, quinine, disopyramide and dopamine [1].

A key element in drug development programs is the measurement of the binding affinities of lead drug candidates for the hOCT1 and the determination whether these compounds are hOCT1 substrates or inhibitors. Currently, cellular uptake studies are used to determine IC_{50} values, which can then be converted to K_is based on the Cheng–Prusoff equation [5]. Although this method provides reliable results, they are time consuming and laborious.

This laboratory has previously developed an alternative method for the study of binding interactions between compounds and receptors or drug transporters [6]. This approach is based upon liquid chromatography utilizing stationary phases containing immobilized membranes from cells expressing the target protein. This program has included the study of the drug exporter P-glycoprotein (Pgp), which is a member of the ABC transporter superfamily [4].

In these studies, membranes from a cell line expressing Pgp and from a cell line that does not express Pgp were immobilized on an immobilized artificial membrane (IAM) liquid chromatographic stationary phase [7–9] or on the surface of a glass capillary to create open tubular columns: Pgp(+)-OT and Pgp(−)-OT [10]. The Pgp-IAM stationary phases were used in frontal affinity chromatography studies to determine the binding affinities (K_d values) of Pgp substrates and inhibitors and were able to identify competitive, allosteric and enantioselective interactions between ligands and the Pgp transporter. The open tubular columns, Pgp(+)-OT and Pgp(−)-OT, were used to differentiate between specific and non-specific interactions between compounds and the immobilized membranes.

In the current study, this experimental approach has been extended to the development of a hOCT1(+)-IAM stationary phase. The column was prepared from a previously described stably transfected MDCK cell line, which expresses hOCT1 [11]. In addition, cellular membranes from the wild-type MDCK cell line were also immobilized onto the IAM stationary phase to produce a hOCT1(−)-IAM stationary phase.

Columns were prepared from both stationary phases and tested to determine the binding activity and specificity of the immobilized hOCT1. The columns were characterized using frontal displacement chromatography with [^{3}H]-MPP$^+$ as the marker ligand and MPP$^+$, verapamil, quinidine, quinine, nicotine, dopamine and vinblastin as the displacers. The results demonstrate that the hOCT1(+) membranes were successfully immobilized on the IAM stationary phase with retention of the ability to specifically bind known hOCT1 ligands and to determine K_d values. The hOCT1(+)-IAM column was also able to determine an enantioselective binding interaction involving (*R*)-verapamil and to identify an additional high affinity MPP$^+$ binding site.

2. Experimental

2.1. Materials

(*R*, *S*)-Verapamil, (*R*)-verapamil, (*S*)-verapamil, *N*-methyl-4-phenyl pyridinium iodide (MPP$^+$), tetraethyl ammonium chloride (TEA), quinine, quinidine, nicotine tartrate, dopamine, vinblastin sulphate, benzamidine, salts, cholate, leupeptin, phenyl methyl sulfonyl fluoride (PMSF), EDTA, Trizma, CHAPS, bovine serum albumin (BSA), glycerol, pepstatin, sodium chloride and dithiothreitol were purchased from Sigma (St. Louis, MO, USA). HPLC grade methanol, ammonium acetate and 0.1 M ammonium hydroxide solution, Whatman GF/C filters were purchased from Fisher Scientific (Pittsburgh, PA). *N*-[^{3}H]-Methyl-4-phenyl pyridinium acetate ([^{3}H]-MPP$^+$) and [^{14}C]-tetraethyl ammonium ([^{14}C]-TEA)) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). 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. Preparation of hOCT1(−)-IAM and hOCT1(+)-IAM stationary phases

2.2.1. Cell lines

The hOCT1(−) membranes were obtained from the MDCK cell line. The hOCT1(+) membranes were obtained from a previously described hOCT1-MDCK cell line [11], which was provided by K. Giacomini (Department of Biopharmaceutical Sciences, University of California San Francisco, San Francisco, CA, USA).

2.2.2. Solubilization of the membranes

The MDCK or hOCT1-MDCK cells (100×10^6 cells) were placed in 15 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 3×30 s at the setting of 12.5 on a Model PT-2100 homogenizer (Kinematica AG, Luzern, Switzerland). The cells were further homogenized in five strokes in a glass Dounce homogenizer (Fisher Scientific, Pittsburgh, PA, USA). The homogenate was centrifuged at $700 \times g$ for 5 min and the pellet containing the nuclear proteins was discarded. The supernatant was centrifuged at $100{,}000 \times g$ for 35 min at 4 °C and the resulting pellet containing the cellular membranes was collected, resuspended in 10 ml of solubilization buffer (Tris–HCl [50 mM, pH 7.4], containing 250 mM NaCl, 1.5% CHAPS, 2 mM DTT, 10 μM PMSF, 8 μM pepstatin A and 10% glycerol) and the resulting mixture rotated at 150 rpm using an orbit shaker (Lab-line Model 3520, Melrose Park, IL, USA) for 18 h at 4 °C.

2.2.3. Immobilization of the solubilized membranes

The resulting solution was centrifuged at 60,000 × *g* for 22 min and the supernatant was mixed with 160 mg of the IAM stationary phase, the resulting mixture was rotated at room temperature for 3 h at 150 rpm using an orbit shaker and then dialyzed against 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 (hOCT1(+)-IAM or hOCT1(−)-IAM) was washed with Tris–HCl [10 mM, pH 7.4] containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and centrifuged. This process was repeated until the supernatant was clear.

2.3. Frontal chromatography with radiolabeled markers

2.3.1. Chromatographic system

The hOCT1(+)-IAM and hOCT1(−)-IAM (180 mg) was packed into a HR 5/2 glass column to yield a 150 mm × 5 mm (I.D.) chromatographic bed. The column was then 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 $MgCl_2$ delivered at 0.2 ml/min at room temperature. Detection of the [^{3}H]-MPP^+ was 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.

2.3.2. Chromatographic studies

The marker ligand used in these studies was [^{3}H]-MPP^+ (20 pM). 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: (*R*, *S*)-verapamil (1, 2, 3, 5 and 10 μM), (*R*)-verapamil (5, 10, 25, 50, 100 and 200 nM), (*S*)-verapamil (1, 2, 5, 10, 15 and 20 μM), quinine (1, 4, 10 and 15 μM), quinidine (1, 3, 5, 20 and 40 μM), nicotine (1, 2, 4, 6, 10, 15, 20 and 30 μM), dopamine (50, 100, 175, 250 and 500 μM), vinblastin (0.5, 1, 2, 5 and 10 μM), MPP high (2.5, 5, 7.5, 10 and 20 pM), MPP low (0.5, 1, 2 and 5 μM).

2.3.3. Data analysis

The dissociation constants (K_d) for the marker and displacer ligands were calculated using a previously described approach [6]. The experimental approach is based upon the effect of escalating concentrations of a competitive binding ligand on the retention volume of a marker ligand that is specific for the target receptor. For example, if the hOCT1 receptor is the target, MPP^+ can be used as the displacer ligand [1,2]. Then, the dissociation constants of MPP^+ (K_{MPP}), as well as the number of the active binding sites of the immobilized hOCT1 receptor (*P*) can be calculated using Eq. (1):

$$[MPP^+](V - V_{min}) = P[MPP^+](K_{MPP} + [MPP^+])^{-1} \quad (1)$$

where *V* is retention volume of MPP^+ and V_{min} the retention volume of MPP^+ when the specific interaction is completely suppressed (this value can be determined by running [^{3}H]-MPP^+ at a high concentration). From the plot of [MPP^+] ($V - V_{min}$) versus [MPP^+], dissociation constant values (K_d), for MPP^+ can be obtained. The same can be done for any other displacer. The data was analyzed by non-linear regression with a sigmoidal response curve using Prism 4 software (Graph Pad Software Inc., San Diego, CA, USA) running on a personal computer.

2.4. Membrane binding assays

The membrane binding assays were carried out as previously described [11]. Briefly, 50 μl of [^{14}C]-TEA (1 μM) was incubated with hOCT1(+) and hOCT1(−) membranes (150 μg/μl) and 50 μl of cold vinblastin (0.5, 1, 5, 10, 50, 100 and 500 μM) in solubilizing buffer (Tris–HCl [50 mM, pH 7.4] containing 250 mM NaCl, 1.5% CHAPS, 2 mM dithiothreitol, 2 μM leupeptin, 2 μM PMSF, 2 μM pepstatin and 10% glycerol) at room temperature for 2 h, before bound and free drug were separated by rapid filtration through Whatman GF/C filters (pre-wetted with solubilizing buffer with 0.1% BSA). The filters were dried and then placed in liquid scintillation vials containing 3 ml of scintillation liquid (Eco-Scint (National Diagnostics, Atlanta, GA) for counting on the Beckman LS60001C liquid scintillation counter (Beckman-Coulter, Fullerton, CA). The data was analyzed by non-linear regression with a sigmoidal response curve using Prism 4 software running on a personal computer.

3. Results

When [^{3}H]-MPP^+ was chromatographed on the hOCT1(−)-IAM and hOCT1(+)-IAM columns, elution profiles containing front and plateau regions were observed for both columns (Fig. 1). The midpoint of the breakthrough curve occurred at 10 min on the hOCT1(−)-IAM column and 17 min on the hOCT1(+)-IAM column, representing breakthrough volumes of 2.0 and 3.4 ml, respectively. Since the void volume of the chromatographic system, column and detector, was 0.7 min, the results indicate that [^{3}H]-MPP^+ was retained on both columns.

The retention of [^{3}H]-MPP^+ on both the hOCT1(+)-IAM and hOCT1(−)-IAM columns is consistent with the observation that [^{3}H]-MPP^+ accumulates in both the hOCT1(+)-MDCK and MDCK cell lines, although the intra-cellular concentration of [^{3}H]-MPP^+ is 10-fold higher in the hOCT1-MDCK cells (data not shown). This suggests that [^{3}H]-MPP^+ interacts with membranes from the MDCK cell line at specific and non-specific sites, other than the hOCT1, and at the same sites plus the hOCT1 with membranes from the hOCT1-MDCK.

The differences in the combinations of specific and non-specific interactions between control and expressed cell

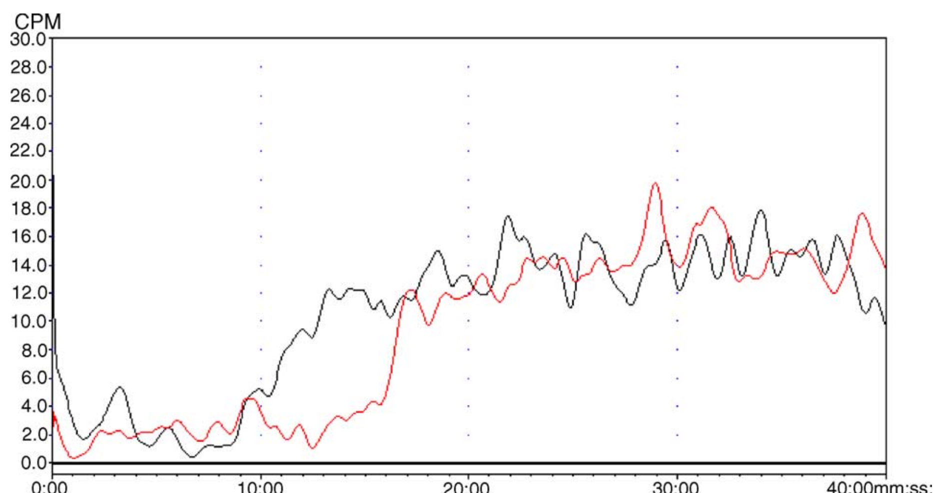

Fig. 1. Comparison of the frontal chromatograms of 20 pM [^{3}H]-MPP$^+$ on the hOCT1(+)-IAM column (red trace) and on the hOCT1-(−)-IAM column (black trace).

lines and the resulting effect on chromatographic retention has been previously demonstrated with the Pgp(+)-OT and Pgp(−)-OT [10]. The studies with the Pgp(+)-OT and Pgp(−)-OT also demonstrated that the specific interactions with the expressed Pgp could be measured using displacement chromatography and Pgp-specific markers.

In this study, the addition of 30 μM nicotine, a competitive inhibitor of the hOCT1 [1], to the running buffer on the hOCT1(−)-IAM column had no effect on the retention of [^{3}H]-MPP$^+$. However, addition of 10 and 20 μM nicotine to the running buffer on the hOCT1(+)-IAM column produced significant and concentration-dependent decreases in [^{3}H]-MPP$^+$ retention (Fig. 2). The results indicate that on the hOCT1(−)-IAM column, the retention of [^{3}H]-MPP$^+$ occurred at sites other than the hOCT1 and that the binding activity of the immobilized hOCT1 could be probed using affinity displacement chromatography.

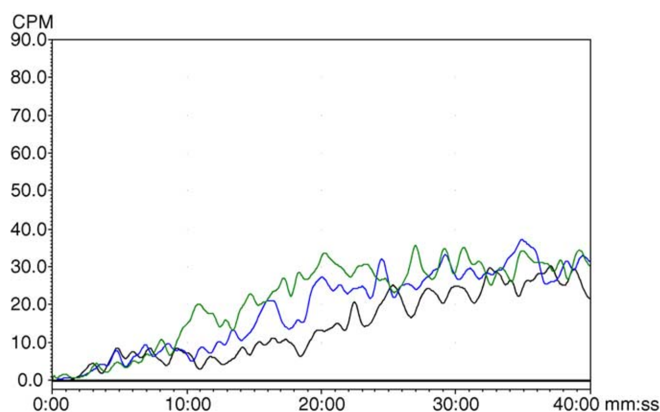

Fig. 2. The effect of the addition of increasing concentrations of nicotine on the chromatographic retention of 20 pM [^{3}H]-MPP$^+$ on the hOCT1(+)-IAM column from no nicotine in the mobile phase (black trace) to 10 μM nicotine in the mobile phase (blue trace) to 20 μM nicotine in the mobile phase (green trace); see Section 2 for details.

The binding activity of the immobilized hOCT1 transporter was determined using frontal displacement chromatography with [^{3}H]-MPP$^+$ as the marker ligand and MPP$^+$, verapamil, vinblastin, quinine, dopamine, nicotine and quinidine as displacers. The competitive frontal displacement of [^{3}H]-MPP$^+$ was seen with all the ligands tested, as illustrated in Fig. 2. Using this approach, the affinity of the displacer for the immobilized hOCT1, expressed as the dissociation constant (K_d) was calculated using Eq. (1) (Table 1). A representative analysis is shown in Fig. 3 (nicotine).

Except for vinblastin, K_i values, obtained from cellular uptake studies, had been previously reported for the test compounds (Table 1). The K_i value for vinblastin (21.48 μM) was determined in this study. In general, the chromatographically determined K_d values were lower than those obtained using membrane binding techniques. In order to determine if the differences represented a qualitative difference between the methods, or a simple quantitative difference, the K_d values obtained by frontal chromatography on the hOCT1(+)-IAM column were compared with those obtained from mem-

Table 1
Binding affinities expressed as K_d values calculated using frontal affinity chromatography on an immobilized hOCT1 column, hOCT1(+)-IAM, compared to K_i values calculated using cellular uptake studies [1,13], with the exception of the value calculated for vinblastin, which was calculated from membrane binding studies conducted during this study

Compound	K_d (μM)	K_i (μM)
(*R*, *S*) Verapamil	2.80 ± 1.09	2.9 [1]
(*R*)-Verapamil	0.05 ± 0.01	Not reported
(*S*)-Verapamil	3.46 ± 1.36	Not reported
Quinidine	6.33 ± 1.48	17.5 [1], 5.4 [13][13]
Methyl phenyl pyridinium	1.80 ± 1.28 $(6.06 \pm 2.87) \times 10^{-6}$	12.3 [1][1]
Quinine	10.18 ± 2.06	22.9 [1]
Nicotine	20.15 ± 6.06	53.2 [13]
Dopamine	198 ± 112	487.2 [13]
Vinblastin	7.28 ± 6.93	21.5

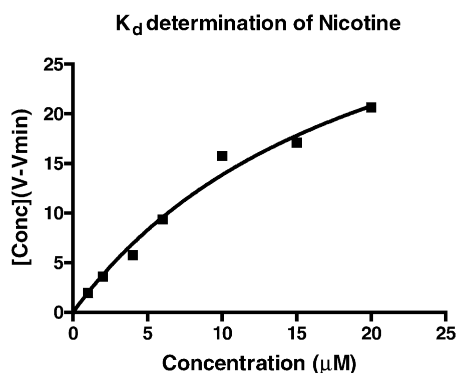

Fig. 3. The relationship between the mobile phase concentration of nicotine and the chromatographic retention volume expressed as $[\mathrm{Conc}](V - V_{min})$ on the hOCT1(+)-IAM column. The data was analyzed by non-linear techniques to determine the binding affinity (K_d) of nicotine for hOCT1 immobilized on the IAM stationary phase.

brane binding studies using a linear regression analysis. A linear relationship was observed with an r^2 value of 0.9987 ($p < 0.001$). To ensure that the observed relationship was not an artifact due to the 40-fold difference between the K_d values of dopamine and the K_d values of the remaining tested ligands, the correlation analysis was carried out without dopamine. A linear relationship was also observed for this data set with an r^2 value of 0.9363 ($p = 0.0016$). Thus, the results indicate that there is only a relative difference between the chromatographically obtained K_d values and those obtained using cellular uptake studies.

In the initial displacement studies of [^{3}H]-MPP$^+$ by MPP$^+$, a low range of displacer concentrations were used (2.5–20 pM). Consistent displacement curves were observed throughout the concentration range and a K_d value of 6 pM was calculated from the data. The result was reproducible on the hOCT1(+)-IAM column used in these studies and on subsequent hOCT1(+)-IAM columns. The calculated K_d value is 10^6 lower than the previously reported K_i value for MPP$^+$ (Table 1).

No additional displacement of the marker was observed until μM concentrations of MPP$^+$ were employed (0.5–5 μM). The analysis from the data from the higher concentration range yielded a K_d value of 1.80 μM, which was consistent with the previously reported value (Table 1).

This appears to be the first time that two binding sites for MPP$^+$ have been identified. This is in agreement with the work by Volk et al. in which high and low affinity binding sites for corticosterone were identified [14]. The different affinities for corticosterone were attributed to the same binding site that underwent conformational changes as a result of applied potential differences. In our studies, we do not immobilize the intact cells, but rather portions of the membranes containing the OCT, therefore the transporter cannot be exposed to a potential differential across the membrane. For this reason, our data represents two separate binding sites for the MPP, which in this case do not overlap. The existence of multiple binding domains on the OCT is also consistent with the previously reported study by Volk et al. [14].

It should be noted that although the K_d value calculated for binding at the high affinity site was 6 pM, this value could be off by an order of magnitude. In order to calculate binding affinities in frontal chromatography, it is assumed that the concentration of the marker ligand is significantly lower than the binding affinity of the marker. Due to the sensitivity of the detector, the marker concentration could not be reduced below 20 pM and thus, the binding affinity at this site could only be approximated. However, in spite of the limitations of frontal chromatography, the hOCT1(+)-IAM column was able to identify a second high affinity site for MPP$^+$, which

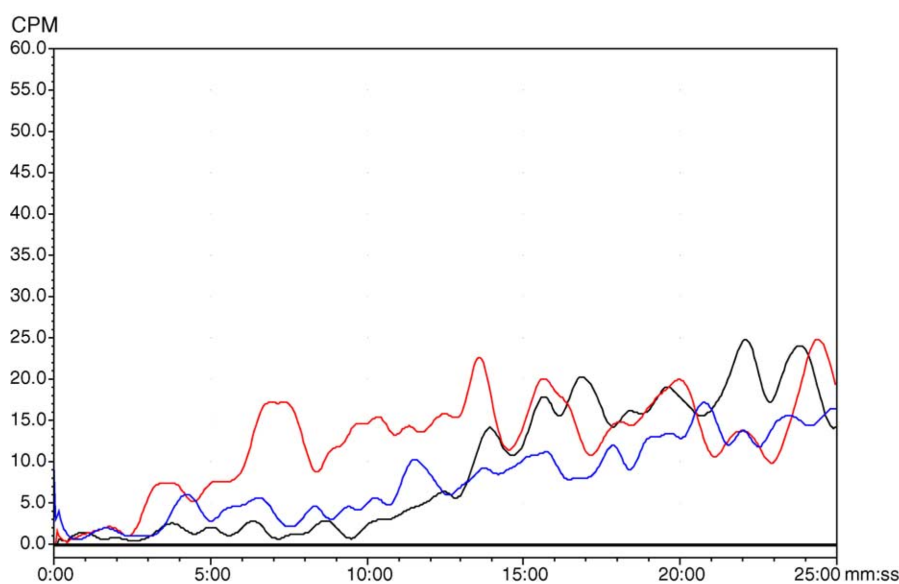

Fig. 4. The effect of the addition of 0.1 μM (*R*)-verapamil (red trace) and 1.0 μM (*S*)-verapamil (blue trace) to the mobile phase on the chromatographic retention of 20 pM [^{3}H]-MPP$^+$ on the hOCT1(+)-IAM column.

had not been observed using membrane binding or cellular transport techniques.

Previous studies have demonstrated that disopyramide enantioselectively inhibited hOCT1-mediated uptake of TEA [12]. In these studies, the IC_{50} value of (*R*)-disopyramide was about two-fold lower than that of (*S*)-disopyramide, 15.4 ± 11.0 and 29.9 ± 8.5 μM, respectively. In this study, (*R*)-verapamil and (*S*)-verapamil were used to investigate the enantioselectivity of the immobilized hOCT1. In the frontal chromatography studies, low concentrations of (*R*)-verapamil produced significant displacements of $[^3H]$-MPP^+ while significantly higher concentrations of (*S*)-verapamil were required to displace the marker (cf. Fig. 4). The results demonstrated that (*R*)-verapamil had a 58-fold lower K_d value than (*S*)-verapamil, 0.05 and 3.46 μM, respectively (Table 1). Since enantiomers have the same physiochemical properties, the observed difference had to be due to specific interactions with immobilized biopolymers. Since no enantioselectivity was observed on the column containing the control membranes, the difference between (*R*)-verapamil and (*S*)-verapamil must be a result of specific interactions with the immobilized hOCT1. Thus, the immobilized transporter retained the ability to enantioselectively bind to substrates and inhibitors. Additional experiments will be carried out to study the enantioselective properties of the hOCT1 transporter and the results will be reported elsewhere.

4. Conclusions

The data from this study indicate that membranes from the hOCT1-MDCK and MDCK cell lines have been successfully immobilized onto the IAM stationary phase, creating hOCT1(+)-IAM and hOCT1(−)-IAM stationary phases. The data also demonstrate that columns containing these stationary phases can be used to determine binding affinities to the immobilized hOCT1 and that the calculated K_d values correlate with K_i values obtained using cellular uptake or membrane binding techniques. In addition, the chromatographic approach can be used to identify binding sites on the hOCT1 and to investigate the enantioselectivity of ligand binding to the transporter.

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