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Keywords:
Blood-brain-barrier; Brain Microvessel Endothelial Cells; Dextromethorphan; Dextrorphan, glutamic acid, NMDA

Abstract:
The mechanisms of uptake and transcellular passage of dextromethorphan (DM) and its major metabolite dextrorphan (DX) across the endothelial component of the blood-brain barrier have been investigated with primary cultures of bovine brain microvessel endothelial cells (BMECs). The uptake of [^{14}C]-DM and [^{14}C]-DX by BMECs was observed to be temperature-sensitive and saturable with approximate K_ms of 0.12 and 0.29 mM, and V_{max}s of 9.2 and 11.0 pmol/mg/min, respectively. The BMEC uptake of [^{14}C]-DM was inhibited half-maximally by approximately 0.57 mM l-glutamic acid, 0.71 mM N-methyl-D-asparatate (NMDA), and 0.99 mM dl-*threo*-β-Hydroxyaspartic acid. The BMEC uptake of [^{14}C]-DX was inhibited half-maximally by approximately 0.48 mM l-glutamic acid, 1.50 mM NMDA, and 0.69 mM dl-*threo*-β-hydroxyaspartic acid. Conversely, the bidirectional passage of DM and DX across confluent BMEC monolayers occurred at a faster rate but was neither saturable nor inhibited by high concentrations of glutamic acid, NMDA, or unlabeled DM or DX. These results suggest that DM and DX were capable of interacting with a low capacity glutamic acid-type carrier mechanism on the apical surface of BMECs. However, the net transfer of these agents across BMEC monolayers appeared to be more rapid and passive in nature.

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Text of paper:

Characterization of Dextromethorphan And Dextrorphan Uptake by a Putative Glutamic Acid Carrier and Passive Diffusion Across Brain Microvessel Endothelium

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RUNNING HEAD: Dextromethorphan and Dextrorphan Uptake

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INTRODUCTION

Stroke is the third leading cause of morbidity and mortality in the United States. Cerebral ischemia is the largest category of stroke (1,2). Ischemic conditions cause impairment of brain energy metabolism and the release of excessive amounts of excitatory amino acids such as glutamate. Under the "excitotoxin hypothesis," high concentrations of glutamate activate calcium channels leading to an influx of calcium and eventual killing of certain populations of neurons in the central nervous system (3,4). Traditional treatments for ischemic stroke have focused on maintaining cardiac output, blood pressure and cerebral blood flow. More recently, attention has been devoted to developing new therapies directed at abnormal biochemical events at excitatory synapses (4-7).

Dextromethorphan (DM) has been generally used as an antitussive in the clinic. Both DM and its de-methylated metabolite dextrorphan (DX) have also been found to be N-methyl-D-aspartate (NMDA) excitatory amino acid receptor antagonists, and antagonists of some calcium channels. A number of studies have demonstrated that DM and DX effectively attenuate both NMDA receptor-mediated neurotoxicity and hypoxic neuronal injury in cortical cell cultures and in cerebral ischemia animal models (8-12). The central nervous system effects of DM and DX presuppose that these drugs are capable of crossing the blood-brain barrier (BBB). To date, however, the precise transport processes regulating passage of DM and DX through the BBB have not been described and have been presumed to be passive in nature.

In this study, we have investigated the cellular mechanisms regulating the uptake and passage of DM and DX across the BBB with primary cultures of brain microvessel endothelial cells (BMECs). Our findings are intended to form a basis for continued exploration into targeted delivery strategies for therapies directed at the treatment of stroke, cerebrovascular disease, and other neurological disorders.

METHODS

Shi, F., Cavitt, J.L., Bailey, C., Malick, A.W., and Audus, K.L. (1993) Characterization of dextromethorphan and dextrorphan uptake by a putative glutamic acid carrier and passive diffusion across brain microvessel endothelium. Drug Delivery 1, 113-118. Publisher's official version: http://dx.doi.org/10.3109/10717549309022764. Open Access version: http://kuscholarworks.ku.edu/dspace/.

Dextromethorphan (DM; *d*-3-methoxyl-17-methylmorphinan), dextrorphan (DX; 17-methylmorphinan-3-ol 9-α, 13-α, 14-α), [^{14}C]-DM (R01-5470; ~50 mCi/mmol) and [^{14}C]-DX (R01-6794; ÷56 mCi/mmol) were provided by Hoffmann-La Roche Inc., Nutley, NJ. Fluorescein (sodium salt), l-glutamic acid hydrochloride, N-methyl-D-aspartic acid (NMDA), glutathione, 2-deoxy-D-glucose, and dl-*threo*-β-hydroxyaspartic acid were purchased from Sigma Chemical Company, St. Louis, MO. All other reagents were of the highest grade commercially available.

Cell Culture and Uptake of [^{14}C]-DM and [^{14}C]-DX

Bovine BMECs were isolated from the gray matter scraped from cerebral cortices as previously detailed by Audus and Borchardt (13,14) and Miller et al. (15). In primary culture, these cells have been characterized biochemically and functionally as an appropriate in vitro model of the endothelial component of the BBB (13-17).

For uptake studies, BMECs (50,000 cells/cm^2) were grown onto rat-tail collagen-coated and fibronectin-treated surfaces in 24-well tissue culture plates (18). Confluent monolayers were formed in ten to fourteen days. To measure the uptake of [^{14}C]-DM and [^{14}C]-DX, cells were first washed three times with warm Hank's balanced salt solution (HBSS) containing 10 mM Hepes buffer, pH 7.4. Monolayers were then incubated with 0.02 µCi/well [^{14}C]-DM or [^{14}C]-DX in a final volume of 1.5 ml (approximately 2.4 µM final concentration of drug) at 37ºC for one minute with or without various concentrations (0.005 - 3 mM) of DM, DX, L-glutamic acid, D-glutamic acid, NMDA or DL-*threo*-β-Hydroxyaspartic acid. Following the incubation, the monolayers were washed three times with ice-cold HBSS buffer, pH 7.4. The monolayers were then incubated in 0.5 ml of 0.25% trypsin-EDTA mixture at 37ºC and solubilized overnight on a Mini Orbital Shaker. The cell lysate was placed in a scintillation vial with 10 ml ScintiVerse E (Fisher Scientific, St. Louis, MO) and cell-associated radioactivities were assayed with a Beckman LS 7500 liquid scintillation counter.

Michaelis-Menten kinetic parameters (e.g., K_m and V_{max}) were estimated from Lineweaver-Burke plots of the difference between 37ºC and 4ºC data. Half-maximal inhibition concentrations (IC_{50}s) for the concentration-dependent effects of selected agents on either DM or DX uptake were estimated from routine nonlinear regression analysis of uptake data.

DM and DX Passage Across BMEC Monolayers

BMECs were grown onto rat-tail collagen-coated and fibronectin-treated polycarbonate membranes (13 mm diameter; 3 µm pore) placed in 100 mm Petri culture dishes (18). The polycarbonate membranes supporting fully confluent BMEC monolayers were placed between side-by-side diffusion cells (Crown Glass C0., Somerville N.J., USA) to monitor the transmonolayer passage of 0.075 µCi of either [14C]-DM or [14C]-DX (approximately 4.5 µM final concentration in drug). Both donor chamber and receptor chamber were filled with 3.0 ml of prewarmed phosphate buffered saline (PBSA; 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 0.63 mM $CaCl_2$, 0.74 mM $MgSO_4$, and 5.3 mM glucose), pH 7.4 as detailed previously (18). The circulating water in the water jacket surrounding the entire diffusion cell was maintained at 37øC. The contents of the diffusion chambers were mixed by a magnetic stir bar set at a constant speed of 600 rpm. A 0.2 ml of sample was taken from the receptor chamber at various times for analysis and a 0.2 ml aliquot of fresh PBSA buffer was added back into the receptor chamber after each withdrawal to maintain volumes. The radioactivities of [^{14}C]-DM and [^{14}C]-DX were assayed with Beckman LS 7500 liquid scintillation spectrometer. Samples were assayed for the presence of fluorescein with an SLM AMINCO Subnanosecond Lifetime Fluorometer (SLM Instruments INC., Urbana, IL), excitation 490 nm and emission 520 nm.

Apparent permeability coefficients were calculated using the formula: $P = X / (A \times t \times C_d)$, where P was the apparent permeability coefficient (cm/sec), A was the diffusion area (0.636 cm^2), and C_d was the concentration of the substance in the donor chamber (mol/cm^3). The flux (mol/cm^2/sec) of a substance

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across the monolayers was calculated from the linearly regressed slope through linear data (e.g., sampling times 1-70 minutes).

Statistics

All uptake and transport experiments were performed in replicates of three to ten different monolayers and data expressed in figures and tables as the mean ± standard deviation (SD). Data were tested for significant differences from untreated controls at the 0.05 level by using an analysis of various (ANOVA; Abstat Software, Ver. 7.01, Anderson-Bell Corp., Arvada, CO).

RESULTS

The BMEC uptake of either DM or DX was concentration-dependent, saturable, and temperature-dependent as shown in Figures 1 and 2. The presence of rather significant cell association of the drugs at low temperature suggests significant cell binding, particularly for DX. In a series of fluorescence polarization experiments (data not shown), we were unable to demonstrate significant interactions of these drugs with BMEC membrane lipids labeled with diphenylhexatriene probes at 37ºC. Therefore, the cell associated drug at low temperature may have been surface binding of DX and DM. Kinetic parameters for DM and DX uptake were estimated from Lineweaver-Burke plots of the difference between BMEC binding of drug at low temperature from the total binding and uptake of drug at 37ºC as shown in insets on Figures 1 and 2, respectively. The V_{max}s were similar and suggested the drugs were using the same uptake mechanism, but differed in affinity for that mechanism.

In a concentration dependent fashion, l-Glutamic acid, NMDA, and dl-*threo*-β-Hydroxyaspartic acid inhibited to some degree the uptake of [^{14}C]-DM and [^{14}C]-DX by BMECs as shown in Figures 3, 4,

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and 5. For both drugs, l-glutamic acid was the more efficient inhibitor of uptake based on estimated IC50s and as summarized in Table 1. Glutathione inhibited uptake to a small degree but only at very high concentrations (>10 mM; not shown).

In contrast to the uptake process, passage of either DM or DX across BMEC monolayers was not saturable over a concentration range of 0.45 µM to 1 mM (not shown). DM crosses the monolayers at a faster rate than DX ($p < 0.05$). Apparent permeability coefficients for the bidirectional passage of DM and DX across the monolayers are listed in Table 2. The corresponding transmonolayer fluxes exceeded the uptake V_{max}s for the drugs (e.g., at 1 µM DM flux ~ 40 pmol/mg protein/min; at 1 µM DX flux ~ 28 pmol/mg protein/min). The difference between the rates of passage of DM from apical-to-basolateral versus basolateral-to-apical was not significant ($p > 0.05$). The difference between the rates of passage of DX from apical-to-basolateral versus basolateral-to-apical was also not significant ($p > 0.05$). Neither DM nor DX had a significant effect on the passage of an impermeant marker, fluorescein, across the BMEC monolayers relative to untreated control monolayers ($p > 0.05$; not shown) and confirmed the absence of possible adverse drug-induced effects on BMEC monolayer permeability.

Passage of DM and DX across the monolayers was not sensitive to 0.01 to 100 mM of selected agents, unlabeled DM, DX, l-glutamic acid or NMDA (not shown). A typical result for l-glutamic acid effects on DX passage across the BMEC monolayers is shown in Table 2. With exception of the basolateral-to-apical passage of DX, a 30 min pretreatment of the cells with a metabolic poison, 50 mM 2-deoxy-D-glucose, also did not significantly alter the passage of either [^{14}C]-DM or [^{14}C]-DX across the monolayers as shown in Table 2.

DISCUSSION

Accumulating evidence suggests that a BBB uptake system for glutamic acid may assist in clearing the amino acid from the central nervous system. Oldendorf and Szabo (19) were the first to

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observe the uptake of glutamic acid and aspartic acid at the BBB. The estimated K_m for glutamic acid uptake at the BBB (20) is at least four times lower than the typical plasma concentration. Consequently, the BBB carrier is normally saturated and does not permit systemic glutamic acid to enter the brain compartment even when systemic concentrations are substantially elevated. The uptake system has been postulated to be part of a protective unidirectional efflux system that provides for the transport of glutamic acid from the brain (20). More recently, Koenig et al. (21) characterized a high affinity NMDA subclass of glutamic acid binding sites on the apical or luminal surface of BBB endothelia. In this latter study, too, the researchers provided evidence that these binding sites may play a role in regulating BBB transport processes.

The mechanism by which DM and DX cross the BBB has been assumed to be passive and has not been extensively investigated either in vivo or in vitro. Although it is clear that these drugs interact with glutamic acid binding sites (5,6), affinity for the amino acid's uptake mechanism has not been investigated. In this study, we have shown that both DM and DX were taken up by BMECs through a saturable and temperature-sensitive system. Several observations here suggest that DM and DX may be interacting with a glutamic acid uptake carrier. First, of those substances tested in this study, glutamic acid was found to be the more effective and complete inhibitor of the BMEC uptake of either DM or DX. Other agents expected to interact with glutamic acid type binding sites, NMDA and DL-*threo*-β-hydroxyaspartate also significantly inhibited DM and DX uptake. The glutamic acid carrier has been described as having one of the lowest transport capacities among the nutrient carriers of the BBB (19,20). The V_{max}s for DM and DX were substantially lower than observed for biotin (18) and leucine (22) carriers in BMEC monolayers and would be consistent with such a low capacity uptake mechanism. The K_m for glutamic acid uptake at the BBB in vivo has not been directly determined, however, Pardridge (20) estimated the apparent K_m from Oldendorf and Szabo's original work (19) to be about 0.04 mM. While the K_ms for DM and DX for the carrier system in this study were higher, it is likely that they were within

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the range one might expect for alternative substrates for the carrier. Finally, the sub-mM concentrations used in this study were consistent with the in vitro pharmacological activity of the drugs in modulating glutamic acid toxicity. DM and DX concentrations of 0.01 to 0.10 mM, for example, inhibit 0.50 mM glutamic acid-induced neurotoxicity in vitro (9). Additionally, DX concentrations of 0.001 to 0.01 mM protect neuronal cell cultures against extracellular acidity (23).

There is at least one other potential carrier known at the BBB for glutamyl-like compounds, a specific and saturable glutathione transcellular transporter (24,25). The glutathione carrier has been described as highly specific, failing, for instance, to recognize either glutathione degradation products or individual amino acids (24,25). In our studies, glutathione was without substantial effects on the BMEC uptake of either DM or DX above the apparent K_m of ÷ 6.0 mM for the glutathione transporter. Thus far, our results are in agreement with the presence of a separate, high affinity, low capacity glutamic acid uptake carrier and suggest that DM and DX may be recognized by a putative glutamic acid uptake system.

Generally, passage of DM and DX across the BBB in vivo has been assumed to be by a passive mechanism. Consistent with that assumption and unlike other agents studied, i.e., biotin (18) and leucine (22), the bidirectional passage of DM and DX across BMEC monolayers was neither saturable nor inhibited by structurally-related agents. The permeation of DM across BMEC monolayers was not substantially altered by pretreatment with the metabolic poison, 2-deoxyglucose, suggesting the absence of any energy-dependence for transmonolayer passage. Since 2-deoxyglucose has been found to reduce passage through the paracellular route for impermeable paracellular markers such as fluorescein and fluorescein-conjugated dextrans (26), results here would also suggest that DM follows a transcellular pathway across the BMEC monolayers. On the other hand, DX passage across the monolayers was reduced by 2-deoxy-D-glucose to a significant degree but not to the extent of fluorescein (26). Based on this observation DX probably utilizes both a transcellular route and any

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paracellular paths that might be available in this in vitro system. Evidence in support of a transcellular route also includes the observation that the fluxes of both DM and DX exceeded the flux of the paracellular marker, fluorescein, across the BMEC monolayers.

In other studies with the BMEC monolayers, we found that for transported substrates such as biotin, the uptake Vmax was within ten-fold of the apparent transmonolayer flux. Differences between the rates could be accounted for by the assymmetrical nature of biotin transfer across the BMECs (18). By contrast, the rates of diffusion of DM and DX across the BMEC monolayers far exceeded the corresponding Vmaxs for apical BMEC uptake and were not significantly different from respective basolateral-to-apical fluxes. Given the absence of factors suggestive of a transcellular carrier mechanism, the large difference between flux and uptake would seem to suggest that the uptake mechanism would play a minor role in the overall transcellular passage of DM and DX.

The rate of DM passage across the BMEC monolayers was significantly greater than DX, perhaps not unlike the situation for their l-analogs. In the heroin-morphine model, masking of hydroxyl groups on morphine with either one acetyl group (i.e., codeine) or two acetyl groups (i.e., heroin) allows enhanced passive permeation across the BBB (27). The removal of the single methyl group from DM to form DX also seems to have limited the availability for transcellular passage and in the process enhanced BMEC binding. It is worth noting that apparent differences in BBB permeability are not necessarily reflective of the pharmacological activity. Despite a lower BBB permeability, morphine is considered the active form of heroin. Similarly, the de-methylated DX is more potent than DM as an anticonvulsant in vivo (5).

In summary, we have provided evidence that DM and DX were taken up on the apical surface of BMECs through a saturable, low capacity carrier mechanism. We suggest that this mechanism may be the glutamic acid uptake carrier of the BBB. Although confirmatory studies are necessary, this observation would suggest that DM and DX could potentially target and modulate the uptake and

biological effects of glutamic acid in the cerebrovasculature. Our results also support the hypothesis that net passage of DM and DX across the blood-brain barrier occurs by a mechanism that is dependent on the physicochemical properties of the individual drugs (i.e., relative lipophilicities) and therefore, passive in nature.

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Table 1. Estimated Half-Maximal Concentrations (IC_{50}s) of Selected Agents that Inhibited Bovine Brain Microvessel Endothelial Cell Uptake of either [^{14}C]-Dextromethorphan ([^{14}C]-DM) or [^{14}C]-Dextrorphan ([^{14}C]-DX) at 37ºC.

	Uptake IC_{50} (mM)	
Agent	[^{14}C]-DM	[^{14}C]-DX
l-glutamic acid	0.57	0.48
N-methyl-D-aspartate	0.71	1.50
dl-*threo*-β-hydroxyaspartic acid	0.99	0.69

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Table 2. Apparent Permeability Coefficients (cm/sec x 10^5) for the Bidirectional Passage of 4.5 µM of either [^{14}C]-Dextromethorphan ([^{14}C]-DM) or [^{14}C]-Dextrorphan ([^{14}C]-DX) across Bovine Brain Microvessel Endothelial Cell Monolayers at 37ºC. Data reported are the means ñ standard deviation from an n = 5.

Drug	Untreated Monolayer	+20 mM l-Glutamic Acid	+50 mM 2-Deoxy-D-glucose
Apical-to-Basolateral Passage			
Dextromethorphan	8.5 ± 0.5 *	-	7.9 ± 0.8
Dextrorphan	6.1 ± 0.8 **	6.9 ± 1.1	5.0 ± 1.9
Basolateral-to-Apical Passage			
Dextromethorphan	9.7 ± 0.4	-	7.1 ± 0.4
Dextrorphan	6.8 ± 0.8	6.6 ± 1.2	4.6 ± 0.4 ***

* Significantly different from dextrorphan ($p < 0.05$) but not different than basolateral-to-apical passage of DM ($p > 0.05$).

** Significantly different from dextromethorphan ($p < 0.05$) but not different than basolateral-to-apical passage of DX ($p > 0.05$).

*** Significantly different from untreated control ($p < 0.05$).

Shi, F., Cavitt, J.L., Bailey, C., Malick, A.W., and Audus, K.L. (1993) Characterization of dextromethorphan and dextrorphan uptake by a putative glutamic acid carrier and passive diffusion across brain microvessel endothelium. Drug Delivery 1, 113-118. Publisher's official version: http://dx.doi.org/10.3109/10717549309022764. Open Access version: http://kuscholarworks.ku.edu/dspace/.

FIGURE LEGENDS

Figure 1. Concentration-Dependent Uptake of [^{14}C]-Dextromethorphan by Bovine Brain Microvessel Endothelial Cells. Filled squares, 37ºC; Open squares, 4ºC; Dashed line, 37ºC with 4ºC data subtracted. Data points represent means ± SD (n = 4). Inset: Lineweaver-Burke plot of dashed line data and kinetic parameters (r^2 = 0.99).

Figure 2. Concentration-Dependent Uptake of [^{14}C]-Dextrorphan by Bovine Brain Microvessel Endothelial Cells. Filled squares, 37ºC; Open squares, 4ºC; Dashed line, 37ºC with 4ºC data subtracted. Data points represent means ± SD (n = 4). Inset: Lineweaver-Burke plot of dashed line data and kinetic parameters (r^2 = 0.99).

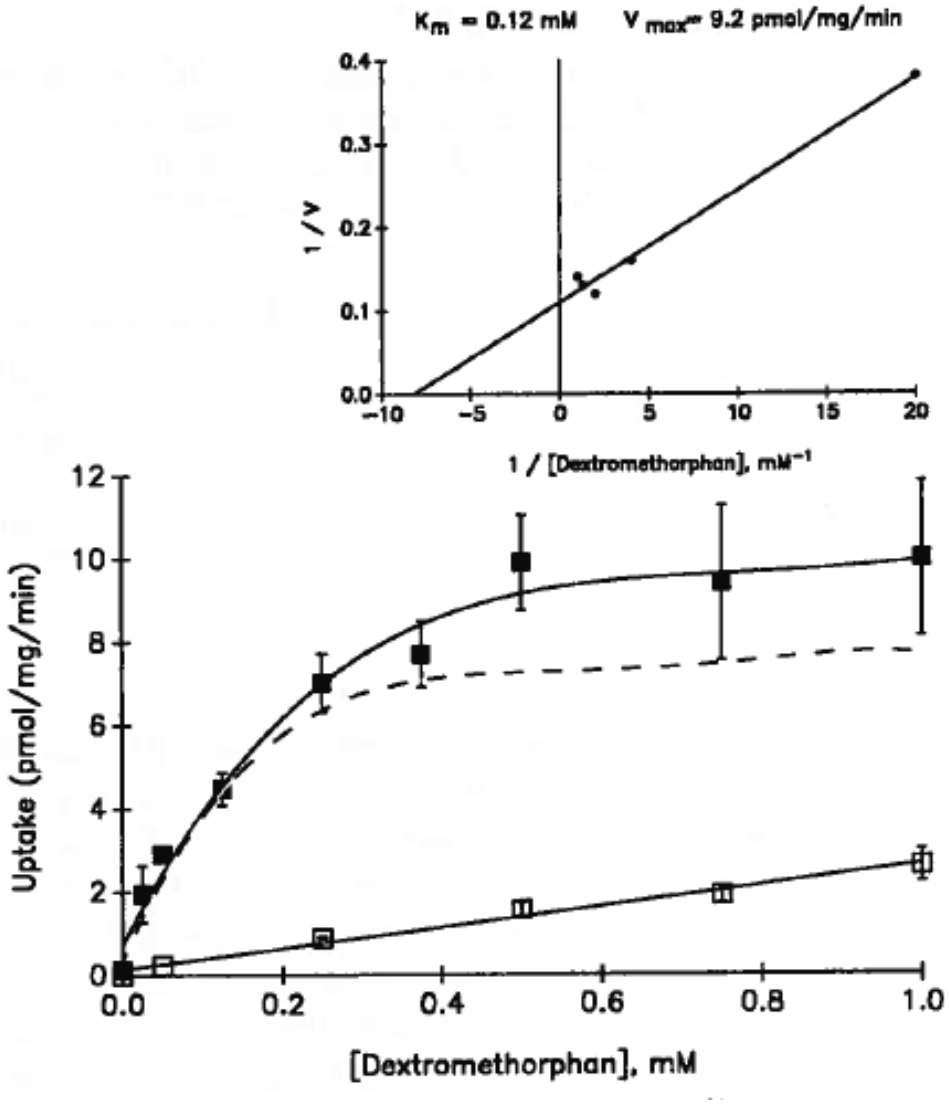
Figure 3. Concentration-Dependent Effect of L-Glutamic Acid on the Uptake of 2.4 µM of either [^{14}C]-Dextromethorphan or [^{14}C]-Dextrorphan by Bovine Brain Microvessel Endothelial Cells at 37ºC. Data points represent the means ± SD (n = 4).

Figure 4. Concentration-Dependent Effect of N-Methyl-D-Aspartate on the Uptake of 2.4 µM of either [^{14}C]-Dextromethorphan or [^{14}C]-Dextrorphan by Bovine Brain Microvessel Endothelial Cells at 37ºC. Data points represent the means ± SD (n = 4).

Figure 5. Concentration-Dependent Effect of dl-*Threo*-β-Hydroxyaspartic Acid on the Uptake of 2.4 µM of either [^{14}C]-Dextromethorphan or [^{14}C]-Dextrorphan by Bovine Brain Microvessel Endothelial Cells at 37ºC. Data points represent the means ± SD (n = 4).

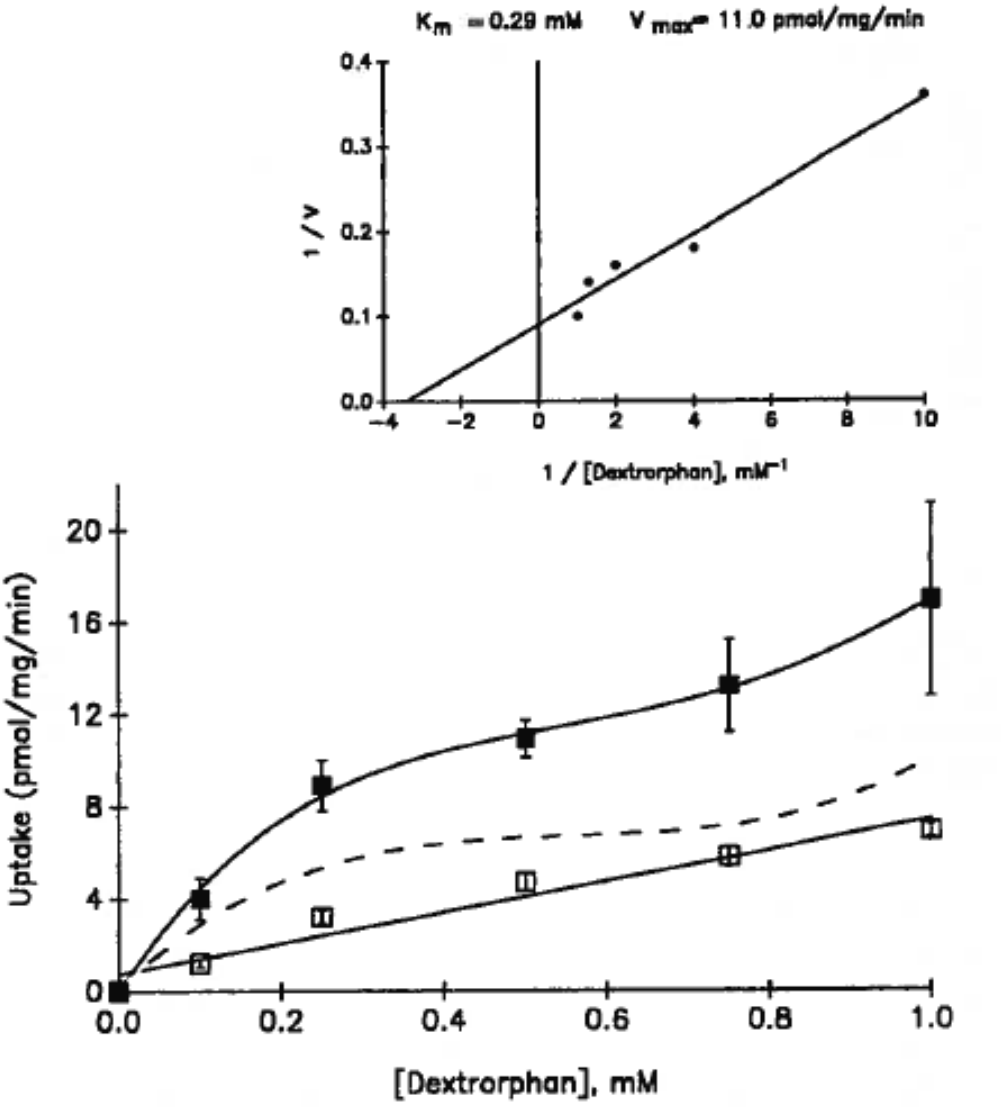
Shi, F., Cavitt, J.L., Bailey, C., Malick, A.W., and Audus, K.L. (1993) Characterization of dextromethorphan and dextrorphan uptake by a putative glutamic acid carrier and passive diffusion across brain microvessel endothelium. Drug Delivery 1, 113-118. Publisher's official version: http://dx.doi.org/10.3109/10717549309022764. Open Access version: http://kuscholarworks.ku.edu/dspace/.

Figure 1



Shi, F., Cavitt, J.L., Bailey, C., Malick, A.W., and Audus, K.L. (1993) Characterization of dextromethorphan and dextrorphan uptake by a putative glutamic acid carrier and passive diffusion across brain microvessel endothelium. Drug Delivery 1, 113-118. Publisher's official version: http://dx.doi.org/10.3109/10717549309022764. Open Access version: http://kuscholarworks.ku.edu/dspace/.

Figure 2



Shi, F., Cavitt, J.L., Bailey, C., Malick, A.W., and Audus, K.L. (1993) Characterization of dextromethorphan and dextrorphan uptake by a putative glutamic acid carrier and passive diffusion across brain microvessel endothelium. Drug Delivery 1, 113-118.
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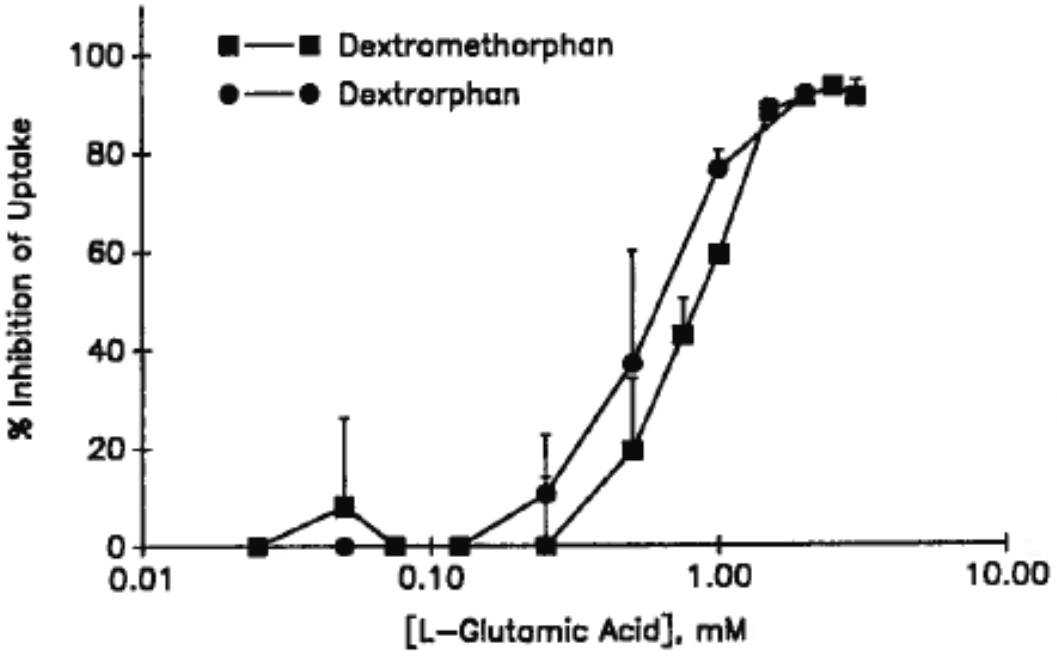
Figure 3



Shi, F., Cavitt, J.L., Bailey, C., Malick, A.W., and Audus, K.L. (1993) Characterization of dextromethorphan and dextrorphan uptake by a putative glutamic acid carrier and passive diffusion across brain microvessel endothelium. Drug Delivery 1, 113-118.
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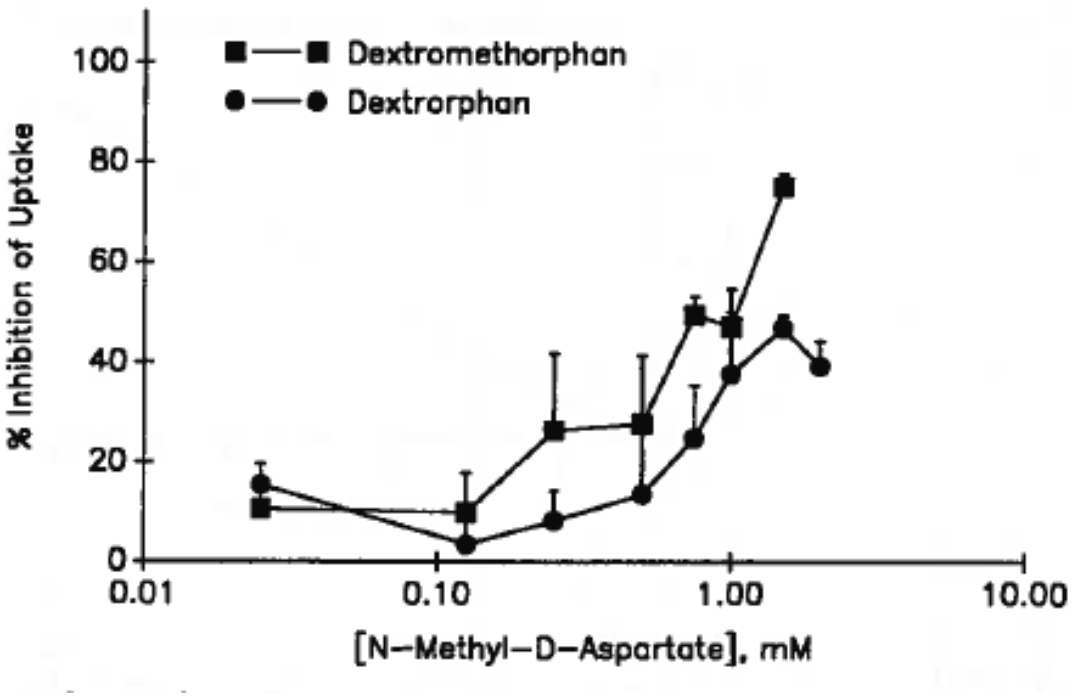
Figure 4



Shi, F., Cavitt, J.L., Bailey, C., Malick, A.W., and Audus, K.L. (1993) Characterization of dextromethorphan and dextrorphan uptake by a putative glutamic acid carrier and passive diffusion across brain microvessel endothelium. Drug Delivery 1, 113-118. Publisher's official version: http://dx.doi.org/10.3109/10717549309022764. Open Access version: http://kuscholarworks.ku.edu/dspace/.

Figure 5