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Life Sciences 77 (2005) 2911–2926

Life Sciences

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Enhancing the uptake of dextromethorphan in the CNS of rats by concomitant administration of the P-gp inhibitor verapamil

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Received 1 June 2004; accepted 30 April 2005

Abstract

Clinical trials evaluating high doses of dextromethorphan hydrobromide (DM) for the treatment of neurological disorders have resulted in numerous adverse events due to the presence of its active metabolite dextrorphan (DX). Since the uptake of drugs in the CNS can be modulated by P-glycoprotein (P-gp) inhibition at the blood–brain barrier (BBB), we propose to determine whether the P-gp inhibitor verapamil can enhance the uptake of DM in the CNS. Rats ($n=42$) received an oral dose of DM (20 mg/kg) alone or 15 min after an intravenous dose of verapamil (1 mg/kg). Rats were euthanized at different time points over 12 h, and concentrations of DM and DX (conjugated and unconjugated) were assessed in plasma, brain and spinal cord using a LC-ESI/MS/MS method. Pharmacokinetic parameters were calculated using noncompartmental methods. Verapamil treatments did not affect the biodisposition of DM in plasma. On the other hand, verapamil treatments increased the area under curve of DM in the brain (from 1221 to 2393 ng h/g) and spinal cord (from 1753 to 3221 ng h/g) by approximately 2-fold. The uptake of DX in brain and spinal cord were markedly lower than those of DM and increased by only 15% and 22% following verapamil treatments, respectively. These results suggest that the P-gp inhibitor verapamil can enhance the uptake of DM in the CNS without affecting that of DX. This change is most likely related to an inhibition of P-gp or other transporters located in the BBB since the biodisposition of DM in plasma remained unaffected by verapamil treatments.

Keywords: Dextromethorphan; Verapamil; CNS; Dextrorphan; P-glycoprotein

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doi:10.1016/j.lfs.2005.04.025

Introduction

Dextromethorphan hydrobromide (DM) is a non-opioid antitussive agent with a wide therapeutic index that has been used for more than 40 years (Bem and Peck, 1992). There has been renewed interest in evaluating the neuroprotective and analgesic effects of DM due to its binding to high- and low-affinity sites and competitive inhibition at the *N*-methyl-D-aspartate (NMDA) receptor in the brain (Steinberg et al., 1993; Advokat and Rhein, 1995). As a result, multiple clinical trials were performed to evaluate the efficacy of high doses DM against neuropathic pain (Sindrup and Jensen, 1999; Sang, 2000; Carlsson et al., 2004), chronic pain (Hewitt, 2000), preoperative and postoperative pain (Weinbroum et al., 2003), Parkinson's disease and dyskinesia (Verhagen Metman et al., 1998; Chase et al., 2000), diabetic neuropathy and postherpetic neuralgia (Sang et al., 2002).

Studies have demonstrated that DM is predominately metabolized via two major pathways. The structure of DM and its demethylated metabolites along with the sites of glucuronidation are presented in Fig. 1. DM undergoes *O*-demethylation by CYP2D1/6 enzymes (i.e., CYP2D1 in rats and CYP2D6 in humans) to dextrorphan (DX) and *N*-demethylation by CYP3A enzymes to 3-methoxymorphinan (3-MM). There is then further demethylation of 3-MM by CYP2D1/6 enzymes and DX by CYP3A enzymes to a third metabolite, 3-hydroxymorphinan (3-OH) (Bochner et al., 1994). DX and 3-MM undergo extensive glucuronidation and approximately 75% of an oral dose of DM is recovered as dextrorphan–glucuronide (DX–Glu) in rats.

The higher doses of DM required for the treatment of various neurological disorder may result in phencyclidine PCP-like side-effects due to extensive metabolism of DM by the CYP2D6 enzyme to its active metabolite DX, a more potent PCP-like non-competitive antagonist at the NMDA receptor than DM (Szekely et al., 1991; Dematteis et al., 1998; Nicholson et al., 1999). The majority of clinical studies evaluating the efficacy of DM administered with a CYP2D6 inhibitor for the treatment of neurological disorders were performed under exploratory conditions in a small number

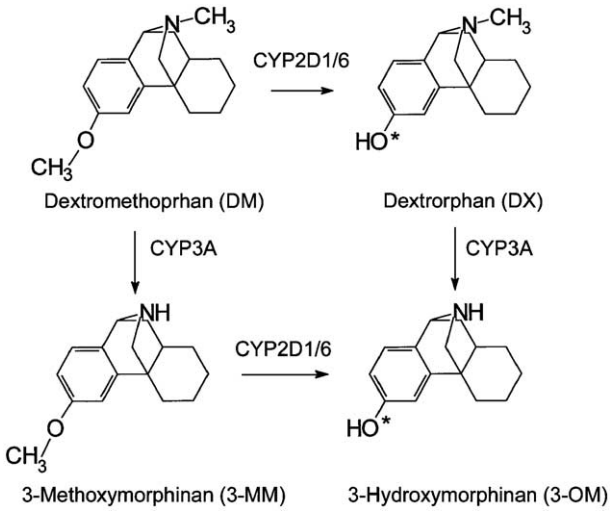

Fig. 1. Metabolic fate and chemical structure of dextromethorphan and its demethylated metabolites. *Glucuronidation sites.

of patients (Schadel et al., 1995; Zhang et al., 1992). Recently, the safety and feasibility of using a CYP2D6 inhibitor to pharmacologically inhibit CYP2D6 activity and increased the systemic bioavailability of DM were evaluated in dose-ranging clinical trials involving a total 121 subjects (Pope et al., 2004). Overall, results of these clinical investigations confirmed that a dose of 25 mg of quinidine was sufficient to suppress *O*-demethylation of DM and that the combination of the two products was safe and well tolerated (Pope et al., 2004). Whereas the concomitant administration of a CYP2D6 inhibitor may improve the therapeutic index of DM by limiting its transformation to DX (Schadel et al., 1995; Zhang et al., 1992; Pope et al., 2004), no research has been performed towards improving the penetrability of DM across the blood–brain barrier (BBB) in order to increase its specific uptake at the site of action.

The BBB is a functional barrier that limits the delivery of numerous compounds to the brain by its physico-anatomical properties and by the presence of ATP-driven drug efflux pumps, such as P-glycoprotein (P-gp), multidrug-resistance associated protein (MRP) and organic anion transporting polypeptides (OATPs) (Elsinga et al., 2004). Results of numerous pre-clinical and clinical studies demonstrated the feasibility of using a P-gp inhibitor to improve drug distribution into the brain (Matheny et al., 2001). More recently, the effect of a P-gp specific inhibitor (valspodar or PSC833) on the brain uptake and analgesic effect of methadone was evaluated in rats (Rodriguez et al., 2004). Results of this study demonstrated that after oral and intravenous administration of methadone, the brain uptake and analgesic effects of methadone in rats pretreated with the specific P-gp inhibitor were increased many-fold (Rodriguez et al., 2004).

On the basis of in vitro and in vivo studies demonstrating that verapamil is a substrate and inhibitor of P-gp and other transporter(s), we postulate that the uptake of DM into the CNS can be enhanced following pre-treatments with verapamil. Since DM displays its neuroprotective effects in the brain and its analgesic effects in the spinal cord, we propose to determine the uptake of DM and DX in these two different regions of the CNS. The objective of the current study was to determine whether an intravenous pre-treatment of verapamil can increase the uptake of DM, DX and DX–Glu in the brain and spinal cord of rats. We also propose to determine whether changes in the uptake of DM and DX in the CNS are driven by any changes in the systemic biodisposition of these products.

Materials and methods

Animals

A total of 42 male Sprague–Dawley rats (Charles River Canada, QC, Canada) weighing between 200 and 300 g were used in this study. Rats were individually housed in polycarbonate cages of conventional design during the acclimation period (1 week) and the conduct of the study. Environmental conditions were monitored during the acclimation period and the conduct of the study (temperature: 21 ± 2 °C; humidity: $50 \pm 20\%$). Rats were fed with a standard certified commercial laboratory diet (Charles River Rodent Chow 5075) and tap water was available ad libitum. The Institutional Animal Care Committee approved the experimental protocol prior to animal experimentation. All experiments were conducted in accordance with guidelines from the Canadian Council on Animal Care and use of laboratory animals.

Dosing

Dextromethorphan hydrobromide (Sigma-Aldrich, Canada) and (*R*,*S*)-verapamil (Sigma-Aldrich, Canada) dosing solutions were prepared in sterile saline (Abbott Laboratories, USA). Male Sprague–Dawley rats either received a single oral dose of 20 mg/kg of DM administered alone or 15 min after a 1 mg/kg intravenous dose of verapamil. Rats were observed following each treatment for any signs of behavioral changes, reaction to test article or ill health. At selected time points (0, 0.25, 0.5, 1, 2, 6, and 12 h) following DM administration and under isoflurane anesthesia, rats were euthanized and intracardiac blood samples were collected in sodium heparin tubes. Blood samples were maintained on ice and centrifuged (3200×*g* at 4 °C for 10 min) within 1 h of collection. Following decapitation, the brain and spinal cord were rapidly removed and frozen in isopentane (−60 °C). The cerebellar and the brainstem tissues were discarded for these analyses. All samples were kept at −20 °C pending analysis of DM and DX.

Analytical methods

Concentrations of DM and DX were determined using an LC-ESI/MS/MS assay. Incubation with β-glucuronidase Type H-1 (Sigma Inc., USA) was used to indirectly measure the glucuronide form of DX (DX–Glu). Dextromethorphan-d_3 and dextrorphan-d_3 were used as internal standards for DM and DX, respectively. To determine brain and spinal cord concentrations of DM and DX, a weighed aliquot of one hemisphere of the brain or spinal cord was added to a buffer solution [0.1 g for 0.075 mL of $NaHPO_4$ (20 mM) and EDTA (1 mM) at pH 7.4] and homogenized.

Briefly, plasma, brain, and spinal cord samples were divided into two 25 μL samples and 25 μL of β-glucuronidase enzyme solution was added to the first sample while 25 μL of enzyme buffer was added to the second sample. The samples were vortexed and incubated at 37 °C for 3 h. After the incubation, 150 μL of the internal standard working solution (dextromethorphan-d_3 and dextrorphan-d_3 in methanol) was added to each sample and the tubes were vortexed. Finally, the samples were centrifuged at 13,000×*g* for 15 min and their supernatants were aliquoted into vials. Samples were injected (5 μL) with a Surveyor LC system (Thermo Electron Corporation, San Jose, CA) onto a Xterra MSC18 analytical column (20×2.1 mm, 3.5 μm; Waters, Milford, MA) with flow rate of 0.5 mL/min. Samples were loaded onto the column with 95% of 0.1% formic acid in water and 5% methanol. After 0.5 min, a linear gradient was applied from 5% to 95% methanol over 2 min. After 0.5 min at 95% methanol, the column was re-equilibrated with 95% of 0.1% formic acid in water for 1 min. The LC cycle time was 4.0 min. The flow from the column was introduced into a Thermo Quantum triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, CA) equipped with an electrospray ionization source. All analytes were monitored in positive mode under MS/MS conditions. The retention times for DM and DX were 1.7 and 2.0 min, respectively. The following instrument parameters were used: sheath gas 30 units, auxiliary gas 15 units, spray voltage 4000 V, and capillary temperature 350 °C. The reactions monitored were: m/z 272→171 (DM), m/z 258→159 (DX), m/z 275→171 (dextromethorphan-d_3), and m/z 261→159 (dextrorphan-d_3). Linearity was assessed by plotting area ratios versus standard concentrations and using a linear regression weighted $1/x$. The correlation coefficients ranged from 0.9946 to 0.9993 for all analytes. Intra- and interbatch precision and accuracy of the assay were assessed with three levels of quality control samples in quadruplicate within each batch. The intrabatch CV% ranged from 1.1% to 12.2% and the interbatch CV% ranged from 3.1% to 10.0%. Intrabatch accuracy ranged from

84.8% to 112.7% and interbatch accuracy ranged from 99.6% to 110.9%. The limit of quantitation (LOQ) of DM and DX were 2.5 ng/mL. DX–Glu concentrations were determined by subtracting the concentrations of the sample incubated with buffer (unconjugated) to the concentrations measured in the sample incubated with β-glucuronidase (total). Concentration values of DM and DX in brain and spinal cord homogenates were corrected by the organ weight and presented as "ng/g" units.

The analytical method for verapamil and norverapamil in plasma consisted of a protein precipitation extraction for sample preparation and a LC-ESI/MS/MS method for analysis. Briefly, 100 μL of the internal standard solution (acetonitrile solution containing 100 ng/mL of terfenadine) was added to 20 μL of plasma samples and the tubes were vortexed. The samples were then centrifuged at 13,000×*g* for 15 min and their supernatants were aliquoted into vials. The supernatants were analyzed using a LC-ESI/MS/MS instrument. Separation was achieved using a Hypersil Phenyl 100 × 2 mm (5 μm) analytical column (Thermo, Electron Corporation, San Jose, CA) with an isocratic mobile phase of acetonitrile and 0.5% formic acid in water (70:30) delivered at a flow rate of 500 μL/min. The mass transition monitored were *m*/*z* 455 → 165 (verapamil), *m*/*z* 441 → 165 (norverapamil), and *m*/*z* 472 → 436 (terfenadine). The collision energy was set to 20 V (E_{lab}) and the declustering potential to 15 V. The nebulization was assisted with nitrogen at a flow rate of 2 L/min and heated at 450 °C. The intrabatch precision results at three levels of quality control samples (low, medium and high) ranged from 0.8% to 8.4%, whereas accuracy results ranged from 91.4% to 104.3%. The interbatch precision at three levels of quality control samples ranged from 2.8% to 5.5%, whereas the accuracy ranged from 97.7% to 105.5%. Mean recovery of verapamil and norverapamil were 88% and 92%, respectively. The analytical range of verapamil and norverapamil were 2.0 to 2000 ng/mL.

Protein binding of DM was determined in triplicate following incubation of 300 ng/mL of DM with increasing concentration levels of verapamil and norverapamil (0, 500, 1000, and 2000 ng/mL) fortified in rat plasma. Following incubation at 37 °C for 60 min, samples were centrifuged in filter cells of 10 kDa (Centricon Plus, Centrifugal Filter Units, Millipore, Billerica, MA) at 3500×*g* for 40 min at room temperature and the ultrafiltrate was aliquoted into vials. The ultrafiltrate was mixed with acetonitrile (1:5) and concentration values of DM were determined by LC-ESI/MS/MS.

Pharmacokinetics and statistical analysis

Concentration–time profiles of DM, DX and DX–Glu in brain, spinal cord and plasma were constructed using the mean concentration values at each time point ($n = 3$ animals per time point for each treatment). Pharmacokinetic parameters of DM, DX and DX–Glu in plasma, brain, and spinal cord were calculated using noncompartmental methods. The area under the curve from time 0 to the last measurable plasma concentration (AUC_{0-t}) was calculated using the linear trapezoidal rule. For the plasma dataset, a rate constant of elimination (k_{el}) was calculated using the last three measurable concentrations and the elimination half-life ($T_{1/2}$) was calculated using $0.693/k_{el}$. The area under the curve extrapolated to infinity (AUC_{0-inf}) was calculated using $AUC_{0-t} + C_{last}/k_{el}$, where C_{last} is the last measurable concentration value in the profile (Rowland et al., 1995). Maximum concentrations (C_{max}) and time to maximum concentrations (T_{max}) in plasma, brain and spinal cord were determined directly on the concentration–time profile. For verapamil only, the predicted plasma concentration at time zero (C_0) was extrapolated by linear regression. Brain-to-plasma and spinal cord-to-plasma ratios of DM, DX and DX–Glu were computed using the appropriate AUC(0–*t*) values. All pharmacokinetic parameters were calculated using Kinetica® Version 4.1.1 (InnaPhase Corporation). Following oral administration

of DM alone, the AUC_{0-t} values of DM, DX and DX–Glu in brain and spinal cord as well as brain-to-plasma and spinal cord-to-plasma ratios were compared to those observed when DM was administered with verapamil using a statistical method involving linear combinations of mean concentrations (Bailer, 1988). Statistical analyses were performed using Microsoft® Excel 2000 for Windows.

Results

Oral administration of DM alone (20 mg/kg) and 15 min after an intravenous dose of the P-gp inhibitor verapamil (1 mg/kg) was well tolerated by male Sprague–Dawley rats. Mean plasma concentration–time profiles of DM, DX and DX–Glu are shown in Fig. 2 (Panels A and B). Mean plasma concentrations of DM peaked at 0.25 h and then declined in a bi-exponential manner. The pharmacokinetic profiles of DM administered alone and with verapamil pre-treatments were superimposable. Mean plasma concentrations of DX were approximately 2-fold lower than those of

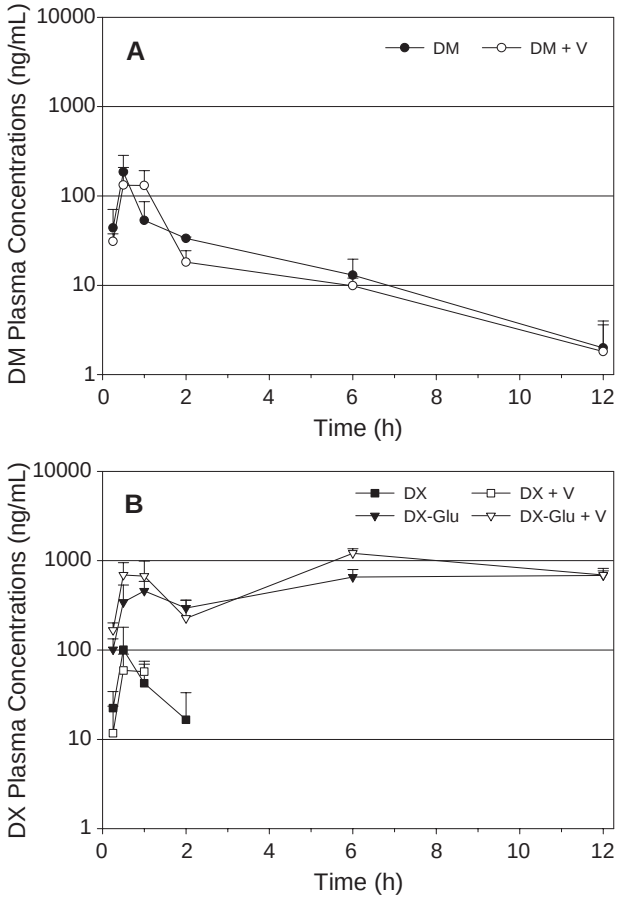

Fig. 2. Mean (+S.E.) concentration–time profiles of DM (Panel A) and DX (Panel B) in plasma (ng/mL) following oral administration of 20 mg/kg of DM alone and 15 min after a 1 mg/kg intravenous dose of verapamil (V) in rats ($n = 3$ animals per time point).

DM and fell below the LOQ after 2 h. On the other hand, mean plasma concentration of DX–Glu were markedly higher than those of DX, suggesting that DX undergoes extensive in vivo glucuronidation in the rat. The sudden increase in plasma concentrations of DX–Glu between 6 and 12 h was attributed to enterohepatic recirculation of DX (Zysset et al., 1988; Schadel et al., 1995). Pharmacokinetic parameters of DM, DX and DX–Glu in plasma are presented in Table 1. The administration of DM with verapamil decreased the AUC_{0-t} of DM and DX in plasma by 7.4% and 47%, respectively. On the other hand, the administration of DM with verapamil increased the AUC_{0-t} of DX–Glu in plasma by approximately 45%. The AUC_{0-t} of DM, DX and DX–Glu in plasma following administration of DM alone were not statistically different than those observed when DM was administered with verapamil.

Mean brain concentrations of DM are shown in Panel A of Fig. 3. Following oral administration of DM alone, brain concentrations of DM peaked within the first hour and then declined in a bi-exponential manner. Concentrations of DM in the brain of rats remained above the LOQ over the whole kinetic study. Administration of DM with verapamil pre-treatments resulted in a marked increase in brain concentrations of DM over the first 2 h and then both concentration–time profiles were superimposable at 6 and 12 h post-dose. Mean brain concentrations of DX and DX–Glu are depicted in Panel B of Fig. 3. Mean brain concentrations of DX were approximately 10-fold lower than those observed for DM. A

Table 1
Mean pharmacokinetic parameters of DM, DX and DX–Glu in plasma following a 20 mg/kg oral dose of DM alone and 15 min after a 1 mg/kg intravenous dose of verapamil in rats

	Treatments	
	DM alone	DM + verapamil
DM		
AUC_{0-t} (ng h/mL)	275	256
AUC_{0-inf} (ng h/mL)	282	263
C_{max} (ng/mL)	185.6	132.0
T_{max} (h)	0.5	0.5
$T_{1/2}$ (h)	2.44	2.95
DX		
AUC_{0-t} (ng h/mL)	83.5	39.4
AUC_{0-inf} (ng h/mL)	NC	NC
C_{max} (ng/mL)	101.0	59.1
T_{max} (h)	0.5	0.5
$T_{1/2}$ (h)	NC	NC
DX–Glu		
AUC_{0-t} (ng h/mL)	6549	9485
AUC_{0-inf} (ng h/mL)	NC	NC
C_{max} (ng/mL)	681.7	1210
T_{max} (h)	12.0	6.0
$T_{1/2}$ (h)	NC	NC

AUC_{0-t}: area under curve from time zero to the last measurable concentration, AUC_{0-inf}: area under curve extrapolated to infinity, C_{max}: maximum plasma concentration, T_{max}: time to maximum plasma concentration, $T_{1/2}$: terminal elimination half-life, NC: not calculated.

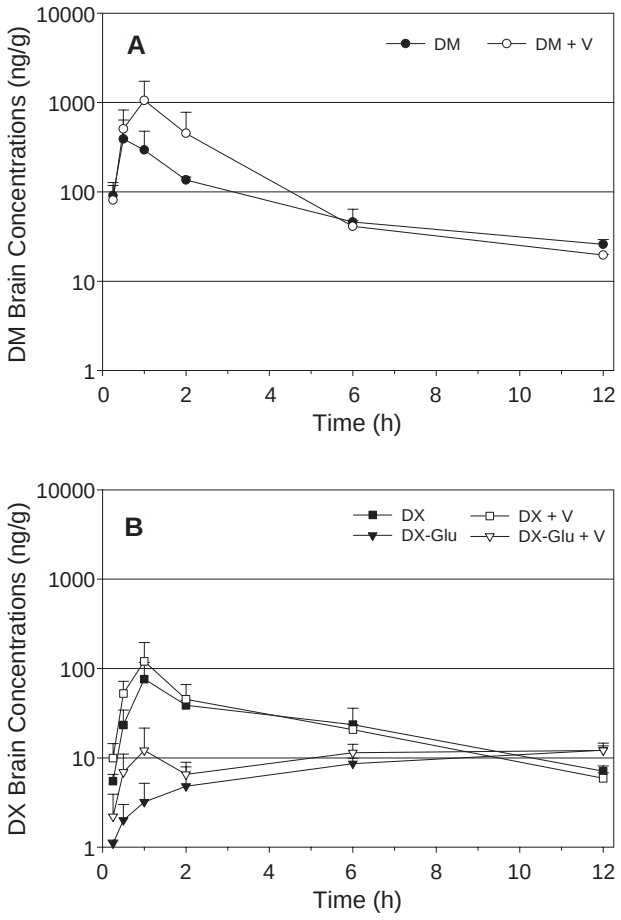

Fig. 3. Mean (+S.E.) concentration–time profiles of DM (Panel A) and DX (Panel B) in brain (ng/g) following oral administration of 20 mg/kg of DM alone and 15 min after a 1 mg/kg intravenous dose of verapamil (V) in rats ($n = 3$ animals per time point).

slight increase in DX brain concentrations was observed over the first hour when DM was administered with verapamil. Mean brain concentrations of DX–Glu were very low and increased marginally when DM was administered with the P-gp inhibitor verapamil. Pharmacokinetic parameters of DM, DX and DX–Glu in brain and brain-to-plasma ratios are presented in Table 2. The administration of verapamil increased the AUC_{0-t} (from 1221 to 2393 ng h/g) of DM in the brain by approximately 2-fold. In contrast to these results, administration of DM with verapamil increased the AUC_{0-t} of DX in its free (from 302 to 346 ng h/g) and glucuronide (from 95.0 to 122 ng h/g) form by 15% and 28%, respectively. The brain-to-plasma exposure of DM was similar to that observed for DX when DM was administered alone (4.4 vs. 3.6, respectively) and with verapamil pre-treatments (9.4 vs. 8.8, respectively). On the other hand, the brain-to-plasma exposure of DX–Glu was markedly lower than that observed for DX when DM was administered alone (0.015 vs. 3.6, respectively) and with verapamil pre-treatments (0.013 vs. 8.8, respectively) due to the marked difference in plasma concentration profiles of DX and DX–Glu. Statistical analysis were performed for brain and brain-to-plasma AUC_{0-t} values of DM, DX and DX–

Table 2
Mean pharmacokinetic parameters of DM, DX and DX–Glu for brain tissues and brain-to-plasma ratio following a 20 mg/kg oral dose of DM alone and 15 min after a 1 mg/kg intravenous dose of verapamil in rats

	Brain		Brain-to-plasma ratio	
	DM alone	DM + verapamil	DM alone	DM + verapamil
DM				
AUC_{0-t} (ng h/g)	1221	2393	4.4	9.4
C_{max} (ng/g)	390.4	1054	2.1	8.0
T_{max} (h)	0.5	1.0	1.0	2.0
DX				
AUC_{0-t} (ng h/g)	302	346	3.6	8.8
C_{max} (ng/g)	75.9	120.3	0.75	2.0
T_{max} (h)	1.0	1.0	2.0	2.0
DX–Glu				
AUC_{0-t} (ng h/g)	95.0	122	0.015	0.013
C_{max} (ng/g)	12.2	19.9	0.018	0.016
T_{max} (h)	12.0	12.0	1.0	2.0

AUC_{0-t}: area under curve from time zero to the last measurable concentration, C_{max}: maximum brain concentration, T_{max}: time to maximum brain concentration.

Glu using the method of Bailer (Bailer, 1988). Considering the small number of animals per time point used in the study and the high variability in brain concentrations, the observed 2-fold increase was not statistically significant at an alpha level of 5%.

DM, DX and DX–Glu were assayed in the spinal cord of rats. Mean concentration–time profiles are depicted in Fig. 4. When DM was administered with the P-gp inhibitor verapamil, mean concentrations of DM in spinal cord displayed similar increases as those observed in brain tissues over the first 2 h. Pharmacokinetic parameters of DM, DX and DX–Glu in the spinal cord of rats and spinal cord-to-plasma ratios are presented in Table 3. The AUC_{0-t} of DM in the spinal cord was approximately 44% higher than that observed in the brain (1753 vs. 1221 ng h/g). The AUC_{0-t} of DM in the spinal cord was increased by approximately 2-fold when DM was administered with verapamil pre-treatments (from 1753 to 3221 ng h/g). This difference was not statistically significant considering the small number of animals per time point and the observed variability in spinal cord concentrations. Verapamil treatments increased the AUC_{0-t} of DX and DX–Glu in the spinal cord by only 22% and 11%, respectively.

Mean concentration–time profiles of verapamil and norverapamil are depicted in Fig. 5. Verapamil treatments were administered 15 min (i.e., −0.25 h, indicated by an arrow in Fig. 5) prior to the oral administration of DM. As a result, the actual sample collection times (0, 0.25, 0.5, 1, 2, 6, and 12 h) were presented relative to the oral administration of DM. Overall, mean plasma concentrations of verapamil declined in a multi-exponential manner and fell below the LOQ at 12 h post-dose. Mean plasma concentrations of norverapamil were markedly lower than those of verapamil. Pharmacokinetic parameters of verapamil and norverapamil are presented in Table 4. When brain and spinal cord concentrations of DM were higher than those observed in control rats (i.e., between 1 and 2 h post-dose), mean plasma concentrations of verapamil and norverapamil ranged from 61.2 to 64.1 ng/mL and 4.5 to 8.6 ng/mL, respectively. The AUC_{0-inf} of verapamil (305 ng h/mL) corresponded to

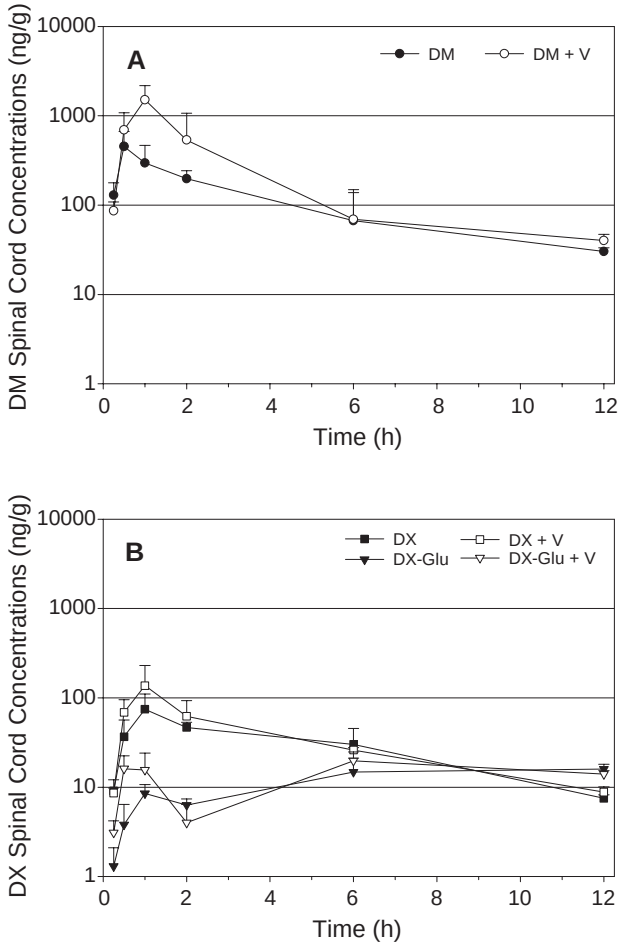

Fig. 4. Mean (+S.E.) concentration–time profiles of DM (Panel A) and DX (Panel B) in spinal cord (ng/g) following oral administration of 20 mg/kg of DM alone and 15 min after a 1 mg/kg intravenous dose of verapamil (V) in rats ($n=3$ animals per time point).

average plasma concentrations (AUC_{0-t}/6 h) of 50.3 ng/mL. In order to determine whether plasma concentrations of verapamil and norverapamil may have affected the free fraction of DM, the binding of DM to plasma proteins was assayed in vitro after incubation with increasing levels of verapamil and norverapamil. Following incubation of 300 ng/mL of DM with 0, 500, 1000, and 2000 ng/mL of verapamil and norverapamil, the protein bindings of DM were 71.6%, 71.3%, 72.5% and 68.2%, respectively.

Discussion

One of the potential problems of using DM for the treatment of various neurological disorders is the PCP-like behavioral side effects associated to its major metabolite DX. Although the concomitant

Table 3
Mean pharmacokinetic parameters of DM, DX and DX–Glu in spinal cord and spinal cord-to-plasma ratio following a 20 mg/kg oral dose of DM alone and 15 min after a 1 mg/kg intravenous dose of verapamil in rats

	Spinal cord		Spinal cord-to-plasma ratio	
	DM alone	DM + verapamil	DM alone	DM + verapamil
DM				
AUC_{0-t} (ng h/g)	1753	3221	6.4	13
C_{max} (ng/g)	452.9	1509.8	2.4	11
T_{max} (h)	0.5	1.0	1.0	2.0
DX				
AUC_{0-t} (ng h/g)	361	440	4.3	11
C_{max} (ng/g)	74.6	136.9	0.74	2.3
T_{max} (h)	1.0	1.0	2.0	2.0
DX–Glu				
AUC_{0-t} (ng h/g)	146	162	0.022	0.017
C_{max} (ng/g)	15.9	19.7	0.023	0.016
T_{max} (h)	12.0	6.0	1.0	1.0

AUC_{0-t}: area under curve from time zero to the last measurable concentration, C_{max}: maximum spinal cord concentration, T_{max}: time to maximum spinal cord concentration.

administration of a CYP2D6 reversible inhibitor may improve the therapeutic index of DM by limiting the formation of DX and increasing its systemic exposure (Schadel et al., 1995; Zhang et al., 1992), no research has been performed towards improving the penetrability of DM into the CNS. P-gp and other transporter proteins present in the brain and spinal cord capillary endothelial cells function to limit cellular exposure to substrates via an active efflux mechanism (Matheny et al., 2001). In this study, we proposed to determine whether an intravenous dose of the P-gp inhibitor verapamil 15 min prior to DM administration can enhance the exposure of DM and DX into the CNS of rats. Whereas research on P-gp

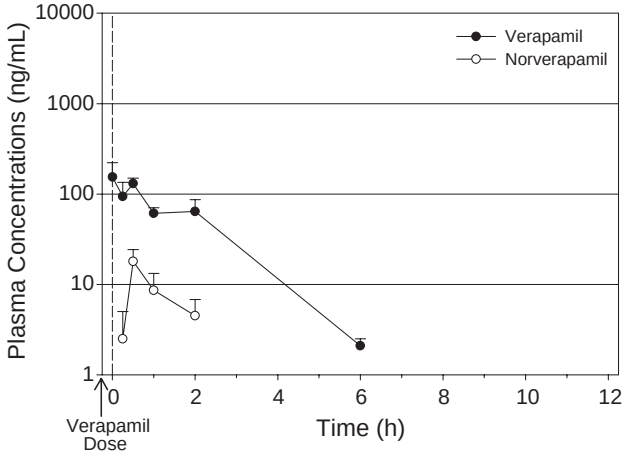

Fig. 5. Mean (+S.E.) concentration–time profiles of verapamil and norverapamil in plasma (ng/mL) following oral administration of 20 mg/kg of DM 15 min after a 1 mg/kg intravenous dose of verapamil in rats ($n = 3$ animals per time point).

Table 4
Mean pharmacokinetic parameters of verapamil and norverapamil following a 20 mg/kg oral dose of DM 15 min after a 1 mg/kg intravenous dose of verapamil in rats

	DM + verapamil
Verapamil	
AUC_{0-t} (ng h/mL)	302
AUC_{0-inf} (ng h/mL)	305
C_0 (ng/mL)	154.3
$T_{1/2}$ (h)	0.950
Norverapamil	
AUC_{0-t} (ng h/mL)	16.1
C_{max} (ng/mL)	18.0
T_{max} (h)	0.5

AUC_{0-t}: area under curve from time zero to the last measurable concentration, AUC_{0-inf}: area under curve extrapolated to infinity, C_0: predicted plasma concentration at time zero, C_{max}: maximum plasma concentration, T_{max}: time to maximum plasma concentration, $T_{1/2}$: terminal elimination half-life.

and other transporter proteins in the brain has showed marked interests over the past few years, only a few researchers have studied their roles in the spinal cord (Sugawara et al., 1990; Koszdin et al., 2000). Since DM exerts an antitussive effect in the medulla region of the brain and an analgesic effects in the spinal cord, we characterized the uptake of DM and DX in these two different areas of the CNS. Finally, we proposed to determine whether changes in the uptake of DM, DX and DX–Glu in the brain and spinal cord were driven by any changes in the systemic biodisposition of these products. Since DX circulates mainly in its glucuronide form after administration of DM in rats, DX–Glu was assessed after incubation with a β-glucuronidase (Zysset et al., 1988; Schadel et al., 1995). Conjugated DX (DX–Glu) was also assayed in brain and spinal cord based on results of other researchers who demonstrated measurable levels of DX–Glu in the brain or rats (Wu et al., 1995).

Following oral administration of DM alone and with verapamil pre-treatments, mean plasma concentration profiles of DM were very similar. Mean plasma concentration of DX–Glu were markedly higher than those of DX, confirming that DX undergoes extensive in vivo glucuronidation in the rat. The sudden increase in plasma concentrations of DX–Glu between 6 and 12 h is likely to be associated to enterohepatic recirculation of DX (Zysset et al., 1988; Schadel et al., 1995). Mean plasma concentrations of DX–Glu increased by approximately 45% following concomitant administration of DM and verapamil. Since DX is mainly eliminated in its glucuronide form in the urine (Zysset et al., 1988), the increase in plasma concentrations of DX–Glu may be attributed to an inhibitory effect of verapamil on the P-gp-mediated renal excretion of DX–Glu.

Peak concentrations of DM in brain of rats were observed within the first hour of drug administration. Following this rapid absorption, DM concentrations declined in a bi-exponential manner and remained above the LOQ (1 ng/mL) in brain for the rest of the kinetic study. Overall, mean concentrations of DM in the spinal cord were approximately 57% higher than those observed in the brain. Although previous studies have characterized the pharmacokinetic of DM in the brain of rats (Char et al., 1992; Wu et al., 1995), this is the first study to present the pharmacokinetic behavior of DM in the spinal cord of rat. The higher availability of DM in the spinal cord may be associated to a higher penetrability in this region of the CNS since the intercellular junctions in the

spinal cord are not as tight as those found in the brain (Sugawara et al., 1990). The overall uptake of DM in the brain and spinal cord of rats, as measured by the AUC_{0-t} of DM and its brain-to-plasma ratio, were increased by approximately 2-fold when DM was administered with verapamil. On the other hand, this 2-fold increase was no statistically significant considering the small number of animals per time point and the high variability in brain concentrations.

The exposure of DX in the brain was approximately 10-fold lower than those of DM. These results suggest that DX may not exert an important pharmacological effect relative to that of DM since its exposure in brain was markedly lower than that of DM. Furthermore, the concomitant administration of the P-gp inhibitor increased the overall uptake of DX in the brain and spinal cord by only 15% and 22%, respectively. When the AUC_{0-t} parameter of DX in brain was adjusted for that observed in plasma, the brain-to-plasma ratio of AUC_{0-t} was similar to that observed for DM when administered alone (4.4 vs. 3.6, respectively) or in combination with verapamil (9.4 vs. 8.8, respectively). Although conjugated DX is not expected to cross the BBB due to its size and hydrophilicity, other researchers have demonstrated measurable levels of DX–Glu in brain tissues of rats (Wu et al., 1995). Overall, concentrations of DX in its glucuronide form were lower than those observed for the unconjugated product in the brain of rats. These brain concentrations are consistent with those previously published by another group (Wu et al., 1995). By considering that unconjugated DX is capable to cross the BBB, it is possible that DX underwent glucuronidation to DX–Glu in situ since high amounts of UDP-GT mRNA were reported to be expressed in the brain of rats (Shelby et al., 2003). On the other hand, amounts of DX–Glu formed into the brain are most likely lower than the circulating amounts in blood capillaries. Specific amounts of DX–Glu into the brain and blood capillaries could not be distinguished in the current study by considering that whole brain of rats were collected and homogenized.

Plasma concentrations of verapamil and its main metabolite norverapamil were assayed using a non-stereoselective method since both the *R*- and *S*-enantiomers of verapamil and norverapamil display similar inhibitory effects on P-gp (Toffoli et al., 1995). Mean plasma concentrations of verapamil and norverapamil corresponding to the time of maximum increase in brain and spinal cord concentrations of DM (i.e., between 1 and 2 h after the intravenous dose of verapamil) ranged from 61.2 to 64.1 ng/mL and from 4.5 to 8.6 ng/mL, respectively. Concentration values for verapamil were approximately in the same range as those observed in clinical settings. For example, repeated oral administration of a 240 mg dose of sustained-release verapamil (Calan SR) in healthy subjects resulted in average plasma concentrations of 68.3 ng/mL over 24 h for verapamil (i.e., AUC_{ss}=1639 ng h/mL) and 89.3 for norverapamil (i.e., AUC_{ss}=2143 ng h/mL) (Abernethy et al., 2000). By considering that the plasma protein binding of verapamil in humans (90%) and rats (95%) are approximately similar and constant over a wide concentration range (Manitpisitkul and Chiou, 1993), then similar effects at the BBB could be expected in a clinical setting.

In vitro determination of plasma protein binding indicated that a change in free fraction for DX and DM by verapamil or norverapamil could not explain the increase in brain/spinal cord distribution. Of all the transporters located at the BBB (P-gp, MRP, OATPs...), P-gp is the most likely candidate involved in the active transportation of DM, by considering that its lipophilicity and chemical structure are similar to those of other opioids (i.e., fentanyl, loperamide, morphine, methadone...) which are transported by P-gp (Matheny et al., 2001; Rodriguez et al., 2004). Moreover, the chemical structure of DM has a cationic nitrogen group, which is a characteristic common to the majority of compounds transported by P-gp (Zamora et al., 1988). On the other hand, our in vivo experimentations did not

allow us to identify the exact protein(s) involved in the active transportation of DM. To establish whether DM is a substrate of P-glycoprotein, transfected cell monolayers, knockout or mutant *mdr1a(−/−)* mice could be used. Considering that the cerebellar and brainstem tissues of rats were discarded in the current study, additional work will be required to determine the preferential distribution of DM, DX and DX–Glu into more specific areas of the brain such as cerebrospinal fluid, right/left hemisphere and endothelium.

Results of this study demonstrate that the administration of DM with an intravenous dose of the P-gp inhibitor verapamil can modulate the uptake of DM in the CNS of rats without affecting that of DX and DX–Glu. By enhancing the specific delivery of DM at the site of action, these results suggest that the administration of DM along with a P-gp inhibitor may improve the therapeutic index of DM for the treatment of neuropathic pain. Provided that multidrug transporters such as P-gp may be involved in the BBB outward transport of drugs, results of this study also suggest that the unintentional combination of DM and a P-gp inhibitor may also lead to an accumulation of DM in the CNS, resulting in unexpected adverse events. This type of drug interaction would be difficult to detect since monitoring of DM and DX in plasma will not allow to detect drug accumulation in the CNS.

Conclusion

Overall, the uptake of DM in the brain and spinal cord was increased by approximately 2-fold when DM was administered with the P-gp inhibitor verapamil. These results suggest that the administration of the P-gp inhibitor verapamil may improve the neuroprotective and anticonvulsant properties of DM since the uptake of DX was not affected. The mechanism underlying this change is most likely related to an inhibition of P-gp or other transporters located in the BBB since the plasma pharmacokinetics of DM remained unaffected by verapamil treatments.

Acknowledgement

We are grateful to the MDS Pharma Services Applied R&D funds and the “Fond du Centenaire de la Faculté de Médecine Vétérinaire” that supported this study.

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