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Determination of the investigational anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid and its acyl glucuronide in Caco-2 monolayers by liquid chromatography with fluorescence detection: application to transport studies

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Abstract

5,6-Dimethylxanthenone-4-acetic acid (DMXAA) is a potent cytokine inducer, with a bioavailability of >70% in the mouse. The aim of this study was to develop and validate HPLC methods for the determination of DMXAA and DMXAA acyl glucuronide (DMXAA-G) in the human intestinal cell line Caco-2 monolayers. The developed HPLC methods were sensitive and reliable, with acceptable accuracy (85−115% of true values) and precision (intra- and inter-assay CV < 15%). The total running time was within 6.8 min, with acceptable separation of the compounds of interest. The limit of quantitation (LOQ) values for DMXAA and DMXAA-G were 14.2 and 24 ng/ml, respectively. The validated HPLC methods were applied to examine the epithelial transport of DMXAA and DMXAA-G by Caco-2 monolayers. The permeability coefficient (P_{app}) values (overall mean ± S.D., $n = 3$–9) of DMXAA over 10–500 μM were independent of concentration for both apical (AP) to basolateral (BL) ($4.0 \pm 0.4 \times 10^{-5}$ cm/s) and BL-AP ($4.3 \pm 0.5 \times 10^{-5}$ cm/s) transport, and of similar magnitude in either direction, with net efflux ratio (R_{net}) values of 1–1.3. However, the P_{app} values for the BL to AP transport of DMXAA-G were significantly greater than those for the AP to BL transport, with R_{net} values of 17.6, 6.7 and 4.5 at 50, 100 and 200 μM, respectively. Further studies showed that the transport of DMXAA-G was Na^+- and energy-dependent, and inhibited by MK-571 [a multidrug resistance associated protein (MRP) 1/2 inhibitor], but not by verapamil and probenecid. These data indicate that the HPLC methods for the determination of DMXAA and DMXAA-G in the transport buffer were simple and reliable, and the methods have been applied to the transport study of both compounds by Caco-2 monolayers. DMXAA across Caco-2 monolayers was through a passive transcellular process, whereas the transport of DMXAA-G was mediated by MRP1/2.

Keywords: 5,6-Dimethylxanthenone-4-acetic acid; Caco-2 cells

1. Introduction

Biological response modifiers act by activating the host's immune functions or modulating other mechanisms such as angiogenesis and metastasis [1,2]. Due to their unusual mode of action, these compounds are often administered chronically and thus oral route is preferred. Among such biological response modifiers, the investigational anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid (DMXAA) was developed as an analogue of flavone-8-acetic acid by the Auckland Cancer Society Research Centre (ACSRC) [3]. DMXAA has multiple pharmacological activities including

Abbreviations: AP, apical; BL, basolateral; CYP, cytochrome P450; DMSO, dimethyl sulphoxide; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; DMXAA-G, DMXAA acyl glucuronide; HBSS, Hank's balanced salt solution; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[4-butanesulfonic acid]; K_i, inhibition constant; K_m, Michaelis–Menten constant; MRP, multidrug resistance protein; 6-OH-MXAA, 6-hydroxymethyl-5-methylxanthenone-4-acetic acid; P_{app}, permeability coefficient; PgP, P-glycoprotein; R_{net}, the ratio of P_{app} in the basolateral to apical direction versus P_{app} in the apical to basolateral direction; TEER, transepithelial electrical resistance; TNF-α, tumour necrosis factor-α; SRB, sulforhodamine B; UGT, uridine diphosphate glucuronosyltransferase; V_{max}, maximal transport rate

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induction of cytokines (in particular tumour necrosis factor (TNF-α)) [4–6], serotonin [7,8] and nitric oxide [9,10], immuno-modulating effects [11,12], NF-κB activation [13], induction of endothelial apoptosis [14], anti-vascular [14,15] and anti-angiogenetic activity [16]. Two recently completed phase I clinical studies on DMXAA involving 109 cancer patients indicated that DMXAA at 1100 and 1300 mg/m^2 gave unconfirmed partial response in two patients [17,18]. DMXAA was well tolerated at lower doses and no drug-related myelosuppression was observed. Rapidly reversible dose-limiting toxicities were observed at 4900 mg/m^2, including confusion, tremor, slurred speech, visual disturbance, anxiety, urinary incontinence and possible left ventricular failure [17,18].

The pharmacokinetic properties of DMXAA have been extensively studied in animals and humans [19–27]. The pharmacokinetics of DMXAA in cancer patients were dose-dependent. Peak concentrations and area under the curve level increased from 4.8 μM and 3.2 μM h, respectively, at 6 mg/m^2 to 1290 μM and 7600 μM h at 3700 mg/m^2, while clearance declined from 7.4 to 1.7 L h^{-1}/m^2 over the same dose range [17]. The terminal half-life was 8.1 ± 4.3 h. DMXAA was extensively bound by plasma proteins (mainly albumin) [28,29]. DMXAA was predominantly metabolized by acyl glucuronidation and to a lesser extent by 6-methylhydroxylation, resulting in DMXAA acyl glucuronide (DMXAA-G) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA), respectively (Fig. 1) [27,30,31]. DMXAA glucuronidation was catalyzed by UGT1A9 and UGT2B7 [23], while 6-methylhydroxylation by CYP1A2 [32]. DMXAA was administered every 3 weeks as a 20-min i.v. infusion in cancer patients [17,18]. The oral bioavailability of DMXAA was about 70% in the mouse, but resulted in

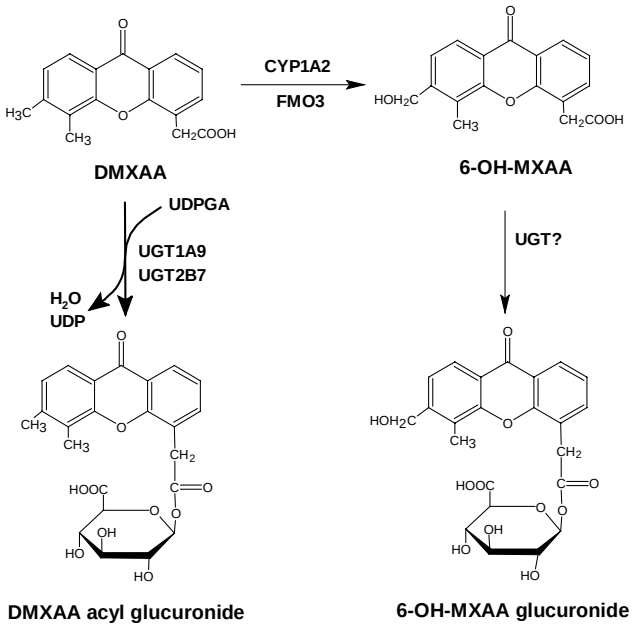

Fig. 1. Metabolic scheme for DMXAA in humans.

low anti-tumor activity [33]. However, when the mice bearing colon cancer 38 xenograft were treated with a loading dose (30 mg/kg) and supplementary doses (15 mg/kg after 4 and 8 h), it gave a 90% cure rate [34]. Thus, oral administration of DMXAA is becoming a possibly effective administration schedule for future clinical trials. In addition, DMXAA has been combined with many drugs such as thalidomide [35], diclofenac [36] and cyproheptadine [37] in the mouse, and these drugs have been shown to modulate the pharmacokinetics of DMXAA. In particular, both thalidomide and cyproheptadine appeared to inhibit the biliary excretion of DMXAA-G in the mouse [37,38]. It is unknown whether modulation of transport of DMXAA and DMXAA-G is involved in these interactions.

Chromatographic methods have been developed and used to quantitate DMXAA and its major metabolites (DMXAA-G and 6-OH-MXAA) in biological matrixes including animal and human plasma, urine and microsomes during pharmacokinetic and metabolism studies [17–27]. These methods involved protein precipitation, sample extraction by liquid–liquid or solid-phase extraction and analysis procedures, which often fulfilled the requirement of quantitation of DMXAA and/or its metabolites in plasma and liver subcellular fractions. Given that DMXAA is a weak acid with a pK_a of 5.5 and present mainly as ionized form at pH 7.4, while DMXAA-G is a highly hydrophilic conjugate with an approximate pK_a of 3.5, active mechanisms may be involved in their intestinal transport. In attempt to characterize the transport of DMXAA and DMXAA-G by the human colon cancer cell line (Caco-2 cells), which have been widely used to investigate drug permeability and transport [39–41], we developed simple HPLC methods for the determination of DMXAA and DMXAA-G in Hank's balanced salt solution (HBSS) used as transport medium. We report here on the developed and validated HPLC methods and their application in the transport studies.

2. Materials and methods

2.1. Chemicals and reagents

DMXAA (98% purity, as determined by thin layer chromatography) and 2,5-dimethylxanthenone-4-acetic acid (SN24350, as internal standard) were synthesized at the Auckland Cancer Society Research center [42]. DMXAA was protected from light exposure to avoid degradation [43]. Authentic DMXAA-G and 6-OH-MXAA were isolated and purified from the bile and urine of rats treated with DMXAA, and their structure confirmed by mass spectrometry and ^{1}H nuclear magnetic resonance (NMR) [31]. Sulforhodamine B (SRB), diclofenac, cyclophosphamide, ifosphamide, cimetidine, probenecid, cephalexin, quinidine, cyproheptadine, and 2-[*N*-morpholino]ethanesulfonic acid were purchased from Sigma–Aldrich (Auckland, New Zealand). ^{3}H-thymidine and ^{14}C-mannitol (specific activity of 5 and 351 mCi/mmol, respectively) were obtained

from Amersham Pharmacia (Auckland, New Zealand). MK-571 was a gift of Dr. Ford-Hutchinson (Merck Frosst Canada, Inc.) [44]. Thalidomide (purity >99%, determined by HPLC) was provided by Celgene Co. (Warren, NJ). Hank's Balanced Salt Solution (HBSS) was prepared by dissolving appropriate solutes in 1 L Milli-Q water [5.95 g *N*-[2-hydroxyethyl]piperazine-*N*′-[4-butanesulfonic acid] (HEPES), 0.14 g $CaCl_2$, 0.40 g KCl, 0.06 g KH_2PO_4, 0.047 g $MgCl_2$, 0.10 g $MgSO_4{\cdot}7H_2O$, 8.00 g NaCl, 0.35 g $NaHCO_3$, 0.048 g Na_2HPO_4, and 4.5 g D-Glucose]. All other reagents were of analytical or HPLC grade as appropriate.

2.2. Cell culture

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained by serial passage in T-75 plastic culture flasks (Life Technologies). The cells were cultured in complete Dulbecco's modified Eagles's medium with 10% fetal bovine serum, 1% non-essential amino acids, and 100 U/ml penicillin and gentomycin (all from Life Technologies). The cells were grown in an atmosphere of 5% CO_2/95% oxygen at 37 °C and given fresh medium every 3 or 4 days. Viable cells were counted using the trypan blue exclusion method. For transport studies, the cells were seeded in 12 mm i.d. Transwell polycarbonate inserts (Corning Costar Corp.) in 12-well plates at a density of 10^5 cells/insert. Cells were used for transport experiments at passage 27–36 at 20–31 days after seeding. The transmembrane specific resistance, expressed in $\Omega\,cm^2$, was measured using a Millicell-ERS apparatus (purchased from Millipore Corporation) at room temperature. The integrity of Caco-2 monolayers was confirmed when the transepithelial electrical resistance (TEER) exceeded 300 $\Omega\,cm^2$, and the leakage of ^{14}C-mannitol was <1%/h.

2.3. Cytotoxicity assay

The cytotoxicity of DMXAA to Caco-2 cells was examined by SRB and ^{3}H-thymidine incorporation assays as described [45,46]. Briefly for the SRB assay, Caco-2 cells were seeded onto 96-well microtiter plates (0.1 ml/well, containing 500 cells/well) and incubated for 24 h at 37 °C. Cells were incubated with DMXAA for 96 h, and cellular proteins were dyed with 0.1 ml 0.4% (w/v) SRB in 1% acetic acid. Cell-bound dye was extracted with 0.1 ml 10 mM unbuffered Tris base (pH 10.5) to solubilise the dye and absorbance determined at 596 nm. For the ^{3}H-thymidine incorporation assay, Caco-2 cells in complete medium were seeded onto 24-well plates (0.5 ml/well, giving 2.5×10^4 cells/well), and incubated for 24 h at 37 °C. Cells were incubated for a further 24 h in low-serum culture medium (with 0.1% bovine serum albumin), followed by DMXAA treatment for 24 h. Two hours before completion of drug exposure, 50 μl (0.5 μCi) ^{3}H-thymidine was added to each well and incubated for 2 h. Cells were then washed with ice-cold PBS and fixed by incubating with 10% trichloride acid at 4 °C for 2 h. Thereafter, the cells were solubilised by incubating with 200 μl 2 M KOH at 55 °C for 30 min, followed by neutralization with 400 μl 1 M HCl. A 300 μl of aliquot was removed for liquid scintillation counting (Beckman Instruments). The IC_{50} value was calculated from concentration–response curves after log/probit transformation.

2.4. Metabolism of DMXAA by Caco-2 cells

For the metabolism study, Caco-2 cells were grown in 60-mm plastic culture dishes. DMXAA (100 μM, total volume 3.7 ml) was added and incubated at 37 °C in triplicate. A 0.5-ml aliquot of medium was collected from each dish at 0, 5, 10, 15, 20, 30 and 60 min for analysis of DMXAA and major metabolites. At the end of the experiments, cells were harvested and digested using 0.02 N HCl/methanol mixture (1:1, v/v), and DMXAA and metabolites determined by HPLC.

2.5. Transport assay

The transport of DMXAA and DMXAA-G by Caco-2 monolayers was investigated using the methods described previously with some minor modifications [47,48]. Briefly, the monolayers were washed twice with warm (37 °C) HBSS containing 25 mM HEPES (pH 7.4) prior to the transport experiments. For apical (AP) to basolateral (BL) transport, DMXAA (10–500 μM) or DMXAA-G (10–200 μM) in transport buffer was added to the AP side (0.5 ml, and 1.5 ml of the transport buffer to BL side). After incubation at 37 °C, an aliquot (0.4 ml) was collected from the basolateral side. The inserts were then removed to new wells containing 1.5 ml fresh HBSS. For BL to AP transport, DMXAA (10–500 μM) or DMXAA-G (10–200 μM) in 1.5 ml transport buffer was added to the BL side, and 0.5 ml of the transport buffer to AP side. The inserts were incubated at 37 °C, and an aliquot (0.4 ml) of sample was collected from the AP side at appropriate times. The incubation medium in the apical side was then replaced with 0.5 ml transport buffer. All incubations were performed in triplicate. To avoid inter-day cell–cell variations, the transport experiments for the determination of kinetics or inhibition by various compounds were always conducted on the same day using the same batch of cells.

The effect of apical or basolateral pH (5.5–7.4) on the AP to BL transport of DMXAA (100 μM) and BL to AP transport of DMXAA-G (100 μM) was examined at pH 7.4 for the receiving side. The pH was altered by substituting appropriate amounts of HEPES in the incubation medium by equimolar (25 mM) 2-[*N*-morpholino]ethanesulfonic acid. In experiments to investigate the effect of Na^+ on the transport of DMXAA or DMXAA-G (100 μM) across the Caco-2 cell monolayers, the NaCl in the HBSS was replaced by equimolar amounts (140 mM) of KCl. The permeability of DMXAA (100 μM) from AP to BL and DMXAA-G from BL to AP

was measured after incubation for 1 h at 4 or 37 °C. To determine energy dependency of DMXAA and DMXAA-G transport, transport medium depleted in glucose was used in both sides of the cell monolayers. Sodium azide (10 mM) or 2,4-dinitrophenol (1 mM) (both ATP inhibitors) was added to both AP and BL side and the monolayers were incubated for 1 h at 37 °C. The inhibitory effects of various compounds (e.g. MK-571 and verapamil) on DMXAA and DMXAA-G transport by Caco-2 monolayers were investigated by adding 100 μM of each inhibitor to both AP and BL side. To obtain hydrolysis products of thalidomide, 100 μM thalidomide in HBSS (1% DMSO, v/v) was incubated at 37 °C for 24 h. All inhibitors were freshly prepared using DMSO immediately prior to experiment and added to the apical side. The final concentration of DMSO in incubations was 1% (v/v), which had no significant effects on TEER values and the transport of DMXAA or DMXAA-G across Caco-2 monolayers. Vehicle (1% DMSO, v/v) was used for the control inserts.

2.6. HPLC

2.6.1. HPLC instrumentation

The Hewlett Packard 1100 HPLC system consisted of an HP G1312A binary solvent delivery system, an HP G1313A autosampler, an HP 1046A fluorescence detector (excitation and emission wavelengths 345 and 409 nm, respectively), and HP1100 ChemStation software for data collection and processing. A Luna C_{18} guard column and a 5 μm Spherex analytical column (150 mm × 4.6 mm; Phenomenex Co., Torrance, CA) were used with a mobile phase (flow rate 1.2 ml/min) of acetonitrile:10 mM ammonium acetate buffer (24:76, v/v, pH 5.0).

2.6.2. Sample preparation

Transport studies were conducted by incubating DMXAA or DMXAA-G with HBSS at either apical or basolateral side of Caco-2 monolayers. An aliquot (400 μl) was collected, mixed with 2 volumes (800 μl) of ice-cold acetonitrile/methonol mixture containing 2% acetic acid (v/v) and 10 μM IS. This protected DMXAA-G from spontaneous degradation. After vortexing for 20 s, the mixtures were centrifuged at 3000 × *g* for 10 min. The supernatant were collected and dried under nitrogen flow using a Speedvac concentrator (Savant Instruments Inc., Farmingdale, NY). The residue was reconstituted with 100 μl mobile phase and 20–50 μl injected onto the HPLC.

2.6.3. Calibration curves

A 500 μM DMXAA (MW = 283) stock solution was prepared by dissolving 70.75 mg of DMXAA in 500 ml methanol, and the stock solution of 268.3 μM DMXAA-G (MW = 459) was prepared by dissolving 0.123 mg of DMXAA-G in 1.0 ml methanol. These stocks were stored at −20 °C. They were than used to make a series of working solutions with HBSS. Calibration samples were prepared by adding known concentrations of DMXAA (0.05–10 and 2.5–100 μM) or DMXAA-G (0.05–10 μM) to HBSS. The ratio of peak area of analyte to that of IS, and linear least-squares regression analysis weighted according to the reciprocal of peak area ratio squares was conducted to determine the slope, intercept and coefficient of determination by Prism 3.0 program (Graphpad Software, CA).

2.6.4. Sensitivity and selectivity

The limit of quantitation (LOQ) was defined as the minimum concentration which could be determined with acceptable accuracy (i.e. recovery between 80 and 120%) and precision (coefficient of variation (CV) <20%) [49]. The limit of detection was the amount which could be detected with a signal to noise ratio of 3. The selectivity of the method was examined by determining if interfering chromatographic peaks were present in blank HBSS or in the presence of various drugs, including thalidomide, diclofenac, verapamil, MK-571, probenecid, and nifedipine.

2.6.5. Accuracy and precision

Quality control (QC) samples containing DMXAA or DMXAA-G at low, medium, and high concentrations were prepared from independent weighings from those used for constructing calibration standards. DMXAA or DMXAA-G was added to HBSS to make quality control samples with concentrations of 0.1, 1, and 10 μM for DMXAA (0.05–10 μM range), 5, 50, and 500 μM (1:10 dilution) for DMXAA (2.5–100 μM range), and 0.1, 1, and 10 μM for DMXAA-G. These samples were prepared on the day of analysis in the same way as standard curves. During each analytical run, QC samples were included and processed as the calibration and unknown samples.

The extraction efficiency for DMXAA (1, 10, and 100 μM) and DMXAA-G (0.1, 1, and 10 μM) from HBSS was determined. The peak areas of both compounds and internal standard extracted from HBSS were compared to those generated by direct injections of both analytes in mobile phase.

2.6.6. Stability of DMXAA and DMXAA-G in HBSS at various pH

DMXAA or DMXAA-G (50 μM) was incubated in HBSS at different pH values (5.4, 6.5, and 7.4) at 37 °C over 6 h. At indicated time points, a 50-μl aliquot of the incubation solution was collected, and acidified by the addition of 2 volumes of ice-cold acetonitrile/methanol mixture containing 2 μM internal standard and 2% acetic acid. After vigorous vortexing and centrifugation at 1500 × *g* for 15 min, the supernatants were collected, dried under nitrogen gas flow using a Speedvac, and then reconstituted with mobile phase, and 50 μl injected into the HPLC.

2.7. Data analysis

Data are presented as mean ± S.D. The apparent permeability coefficients (P_{app}), expressed in cm/s, were calculated

by the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{60} \times \frac{1}{A} \times \frac{1}{C_0} \quad (1)$$

where $\Delta Q/\Delta t$ is the permeability rate (nmol/min); A the surface area of the membrane (cm^2); and C_0 is the initial concentration in the donor chamber (μM). The net BL to AP efflux of DMXAA or DMXAA-G (R_{net}) was determined by calculating the ratio of P_{app} in the BL to AP direction versus P_{app} in the AP to BL direction ($P_{app\,BL-AP}/P_{appAP-BL}$) as Eq. (2) [50,51].

$$R_{net} = \frac{P_{app\,BL-AP}}{P_{app\,AP-BL}} \quad (2)$$

The passive diffusion flux rate (excluding the influence of efflux transporter) of DMXAA-G in Caco-2 monolayers was estimated by conducting the transport experiment in the presence of MK-571 (50 and 100 μM). The active transport flux rates were then estimated by subtracting the passive diffusion rates from total flux rates. Several models to describe the kinetics of the active transport of DMXAA-G (single and two binding sites, substrate inhibition, and the sigmoid models) were fitted and compared using the Prism 3.0 Program (Graphpad Software, CA). The choice of model was confirmed by F-test and Akaike's information criterion [52]. It was found that one-binding site model (Eq. (3)) was the best fit.

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (3)$$

where v is the apparent linear initial rate, $[S]$ the initial concentration, V_{max} the maximum transport rate, and K_m is the Michaelis–Menten constant.

Apparent inhibition constant (K_i) was estimated using Eqs. (4)–(6) as previously described [50,53].

$$K_i = \frac{P_I/P_0}{1-(P_I/P_0)}[I] \quad (4)$$

$$P_0 = P_{app1} - P_{app3} \quad (5)$$

$$P_I = P_{app2} - P_{app3} \quad (6)$$

where P_I and P_0 are the P_{app} values of DMXAA-G in the direction of BL to AP in the presence and absence of the inhibitor, respectively; and P_I/P_0 is a reflection of the inhibitory effect of the test compound on the active BL to AP transport of DMXAA-G across the Caco-2 monolayers. $[I]$ is the concentration of inhibitor in the donor and the receiver side. P_{app1} is the total transport in the absence of any inhibitory compound; P_{app2} the total transport in the presence of a potential inhibitor; and P_{app3} is the passive diffusion component.

The initial statistical analysis to evaluate the differences in the mean kinetic parameters among the different groups was performed by a one- or two-way analysis of variance (ANOVA) with a post-hoc test (Dunnett's multiple comparison test). Student's unpaired t-test was conducted for between-group comparison. $P < 0.05$ was regarded as significance.

3. Results

3.1. Validation of HPLC methods

Representative chromatograms for DMXAA and DMXAA-G with internal standard in HBSS are shown in Fig. 2. Under the chromatographic conditions described above, the retention times for DMXAA, DMXAA-G and IS were 4.8, 1.7, and 6.1 min, respectively. Matrix-specific interfering peaks that required modification of the mobile phase composition were not observed. The presence of drugs such as thalidomide, diclofenac, verapamil, MK-571, probenecid, and nifedipine also did not cause any interference in the assay.

Calibration curves for DMXAA were linear over the concentration range of 0.05–10 and 2.5–100 μM with the mean correlation coefficients >0.999 ($n = 9$ and 7, respectively). The mean y-intercepts were 0.009 ± 0.002 ($n = 3$) and 0.05 ± 0.008 ($n = 4$), and the mean slopes were 0.49 ± 0.008 ($n = 3$) and 0.128 ± 0.002 ($n = 4$), for DMXAA in HBSS

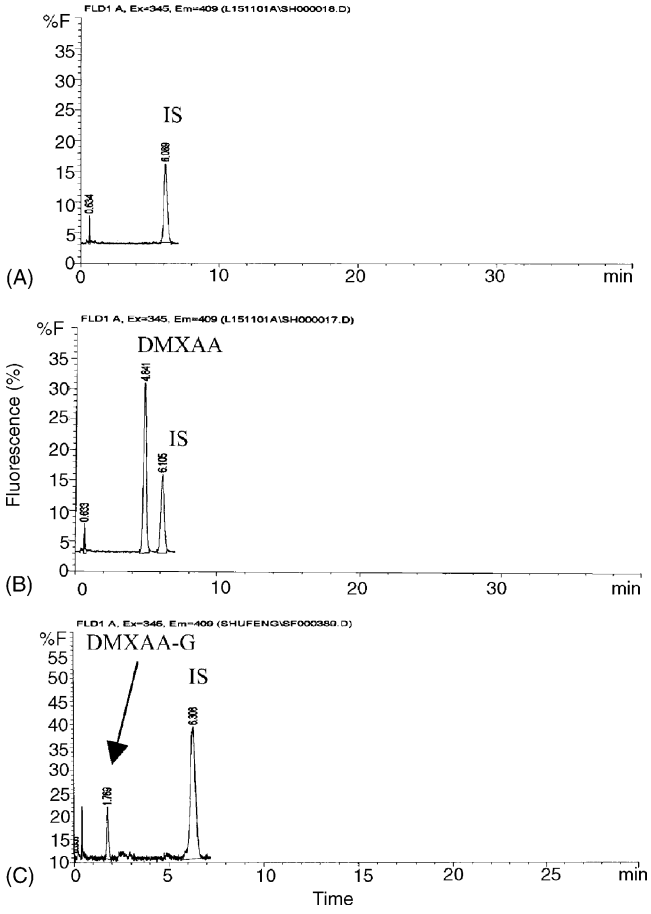

Fig. 2. Representative chromatograms for blank HBSS spiked with (A) 10 μM internal standard (SN24350, IS) (B) 2.5 μM DMXAA and internal standard, and (C) 0.5 μM DMXAA-G and internal standard.

Table 1
Accuracy and precision of the HPLC method for the analysis of DMXAA and DMXAA-G in transport buffer (HBSS)

Theoretical concentration (μM)	Measured concentration (μM) (mean ± S.D.)	Percentage recovery of theoretical	CV (%)	Number of samples
DMXAA				
Intra-assay				
0.1	0.095 ± 0.009	95.2	9.8	4
1	0.99 ± 0.02	99.1	2.3	4
10	10.1 ± 0.3	100.6	3.4	4
5	4.9 ± 0.4	98.7	7.4	4
50	48.9 ± 2.6	97.8	5.2	4
500	485.1 ± 13.6	99.0	2.8	4
Inter-assay				
0.1	0.09 ± 0.01	93.3	14.9	4
1	1.00 ± 0.08	100.3	8.3	4
10	9.9 ± 0.9	98.7	9.3	4
5	4.9 ± 0.7	98.4	14.8	4
50	49.9 ± 6.2	99.8	12.5	4
500	496.4 ± 99.3	99.3	5.7	4
DMXAA-G				
Intra-assay				
0.1	0.11 ± 0.007	111.3	6.6	4
1	0.99 ± 0.06	99.4	5.9	4
10	10.0 ± 0.2	100.2	1.9	4
Inter-assay				
0.1	0.106 ± 0.015	105.7	14.7	4
1	0.98 ± 0.09	98.4	8.8	4
10	10.2 ± 0.4	101.8	4.0	4

at 0.05–10 μM and 2.5–100 μM, respectively. Calibration curves for DMXAA-G were linear over the concentration range of 0.05–10 μM with the mean correlation coefficients >0.999 ($n = 9$). The mean y-intercepts were 0.006 ± 0.002 ($n = 3$) and the mean slopes were 0.33 ± 0.03 ($n = 3$).

The differences between the theoretical and the actual concentration and the relative standard were less than 15% at all QC concentrations. The results of the precision and accuracy were shown in Table 1. The extraction efficiency for DMXAA was 96.5 ± 6.8, 97.6 ± 8.9, and 97.9 ± 7.2 ($n = 3$) at concentration of 1, 10, and 100 μM, respectively. No concentration dependence was observed. Extraction efficiency for DMXAA-G was 94.5 ± 6.1, 92.9 ± 6.9, and 91.9 ± 7.1 ($n = 3$) at concentration of 0.1, 1, and 10 μM, respectively. No concentration dependence was observed.

The LOQ was evaluated based on the precision and accuracy of the assay performed. Below 0.05 μM for DMXAA and DMXAA-G in HBSS, the accuracy and precision of the HPLC methods were not acceptable, when the injection volume was 50 μl; therefore this concentration was the LOQ for both compounds (i.e. 14.2 and 24 ng/ml for DMXAA and DMXAA-G, respectively).

As shown in Fig. 3A, DMXAA was stable over 6 h in HBSS at pH 5.5–7.4. However, DMXAA-G in HBSS at pH 7.4 was unstable, with a degradation half-life of 2.5 h. At pH 7.4 after 1 h, 88% of DMXAA-G remained unchanged (Fig. 3B). When the pH was reduced to 6.6, the degradation half-life increased to 10.3 h. No significant degradation was observed at pH 5.5.

3.2. Cytotoxicity and metabolism of DMXAA in Caco-2 cells

The inhibition of Caco-2 cell growth by DMXAA using SRB assay and the effect of DMXAA on DNA synthesis inhibition of Caco-2 cells are shown in Fig. 4. DMXAA at concentrations ≤250 μM had negligible inhibitory effect on the growth of Caco-2 cells. However, with concentrations ≥500 μM, significant inhibition was observed ($P < 0.05$), with an IC_{50} value of 785 ± 65 μM. Similarly, DMXAA inhibited the ^{3}H-thymidine incorporation by Caco-2 cells in a concentration-dependent manner, with an IC_{50} value of 735 μM. It appeared that ^{3}H-thymidine incorporation assay was more sensitive than SRB assay.

There was detectable DMXAA-G and 6-OH-MXAA in the culture medium following addition of 100 μM DMXAA to Caco-2 cells (Fig. 5). There was a slow increasing formation of the two major metabolites over 60 min, accounting for 5.0% DMXAA disappearance. There were no detectable metabolites within the Caco-2 cells. DMXAA concentration in the medium remained unchanged within 20 min, with a 5–10% decrease by 60 min.

3.3. DMXAA and DMXAA-G transport by Caco-2 monolayers

The time course of transport of DMXAA (10–500 μM) from AP to BL or BL to AP is shown in Fig. 6A and B. After apical or basolateral loading, DMXAA appeared on the

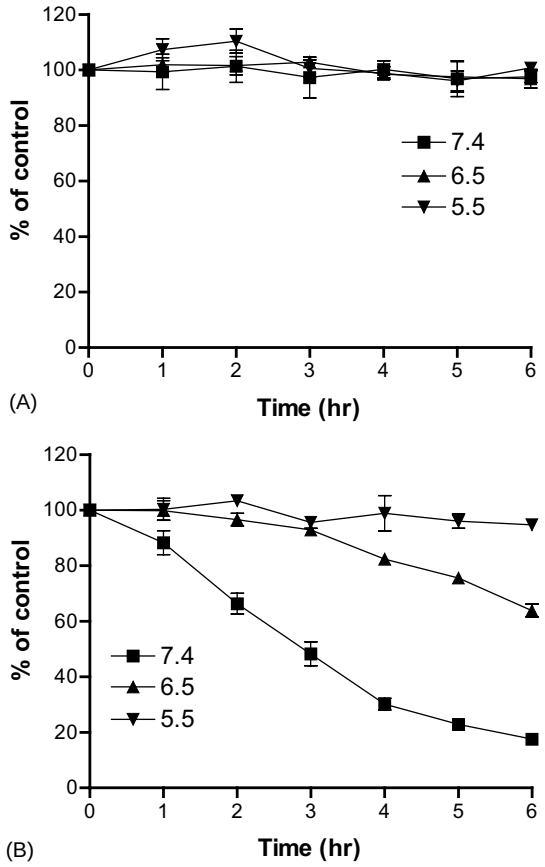

Fig. 3. Stability of DMXAA (A) and DMXAA-G (B) in HBSS at pH 5.5, 6.5 and 7.4. DMXAA or DMXAA-G (both at 50 μM) was incubated in HBSS at various pH values, and aliquots were collected over 6 h for the analysis of DMXAA or DMXAA-G.

receiving side by 15 min. The AP-BL and BL-AP transport amounts (nmol) of DMXAA at 10–500 μM were of similar magnitude and appeared to be linear over the initial 2 h. However, it was noted that there appeared to be a faster transport (approximately three-fold) of DMXAA in the AP to BL

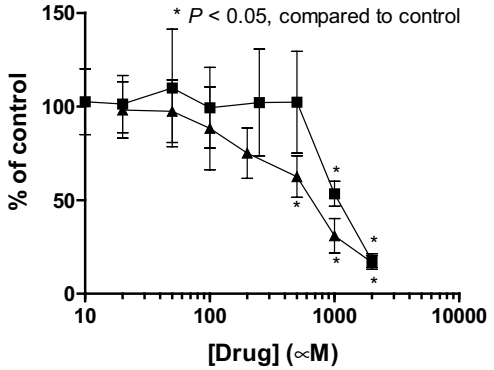

Fig. 4. Effects of DMXAA on the growth and DNA synthesis of Caco-2 cells using SRB assay (■) and ^{3}H-thymidine incorporation assay (▲). Data were the mean ± S.D. from six determinations.

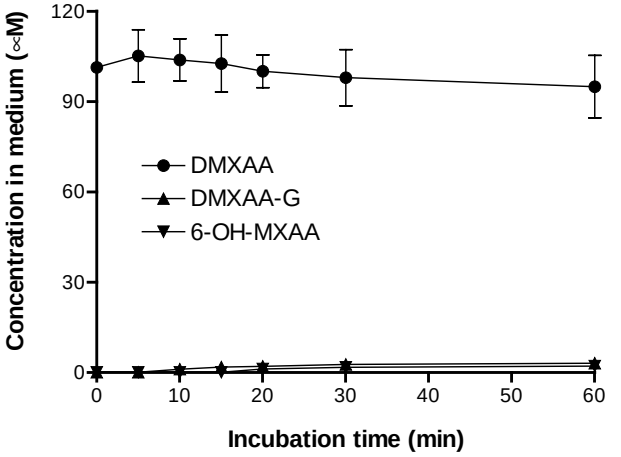

Fig. 5. Metabolism of DMXAA (100 μM) incubated with Caco-2 cells at 37 °C. At indicated times, a 0.4-ml aliquot was collected and DMXAA, DMXAA-G and 6-OH-MXAA were determined by HPLC. Data were the mean ± S.D. from three determinations.

direction during the initial 15 min period with DMXAA concentration ≥300 μM. The transport rates (nmol/min/cm^2) of DMXAA from AP to BL, or BL to AP, were similar and were directly proportional to DMXAA concentrations over 10–500 μM (Fig. 7A). No detectable DMXAA-G and 6-OH-MXAA were observed when DMXAA was loaded on apical or basolateral side at all concentration. The P_{app} values (overall mean ± S.D., n = 3–9) of DMXAA were independent of concentration for both AP-BL (4.0 ± 0.4 × 10^{-5} cm/s) and BL-AP (4.3 ± 0.5 × 10^{-5} cm/s) transport, and of similar magnitude in either direction, with R_{net} values of 1–1.3 (Fig. 7B).

As shown in Fig. 6C and D, when DMXAA-G at 10 and 25 μM was loaded on the apical side, there was no detectable DMXAA-G on the receiving side. At concentrations of DMXAA-G ≥50 μM, the AP to BL flux was essentially linear for up to 1 h in a concentration-dependent manner with no apparent saturation. The transport rate of DMXAA-G from BL to AP was 5–10-fold faster than that of AP to BL direction, with measurable amounts of DMXAA-G appearing on receiving side even at low loading concentrations (10–25 μM) (Fig. 7C). The BL to AP transport of DMXAA-G increased linearly with increasing concentration, with a suggestion of saturation at concentrations ≥ 50 μM. The P_{app} values (0.03–0.04 × 10^{-5} cm/s) for AP to BL flux of DMXAA-G over 50–200 μM were independent of concentration (P > 0.05) (Fig. 7D). However, the P_{app} values for the BL to AP transport of DMXAA-G were significantly greater than those for the AP to BL transport, with R_{net} values of 17.6, 6.7 and 4.5 at 50, 100 and 200 μM respectively (Fig. 7C). The BL to AP transport of DMXAA-G was saturable, as indicated by the significant decrease in P_{app} at DMXAA-G concentrations ≥50 μM (P < 0.001). Model fitting indicates that the BL to AP active efflux followed one binding-site kinetics, with a K_m of 83.5

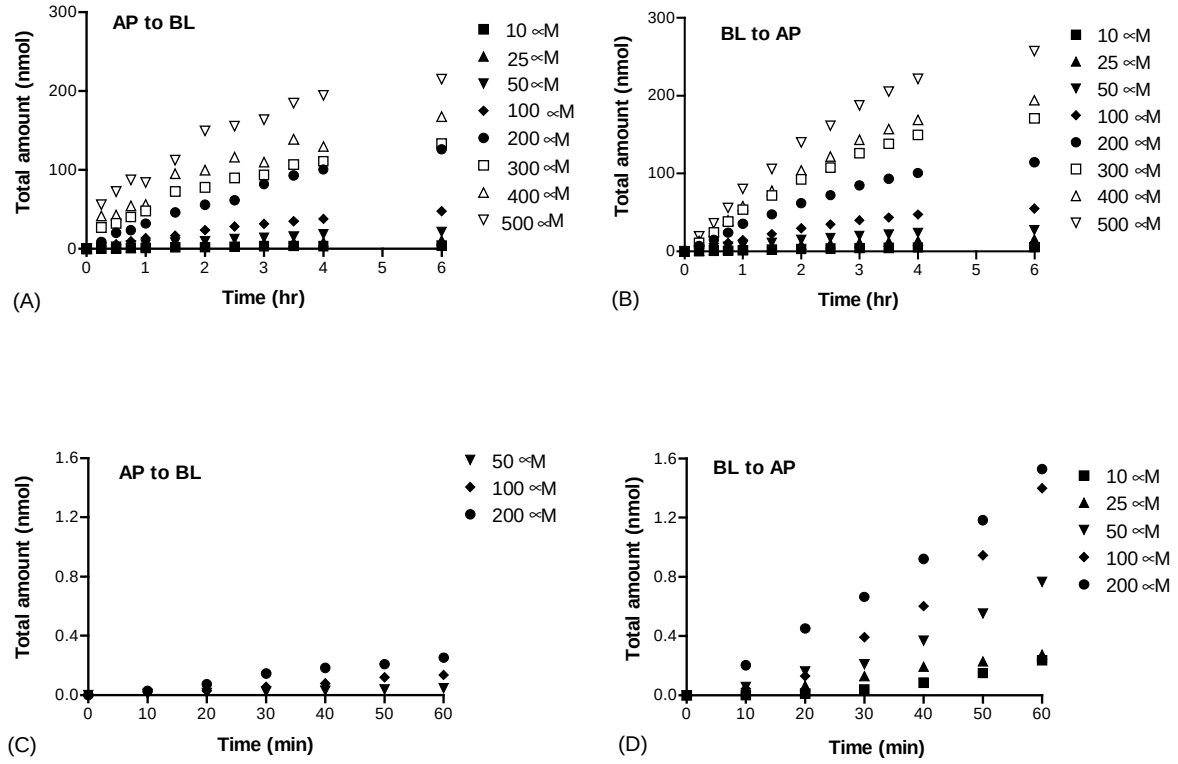

Fig. 6. Time course for apical (AP) to basolateral (BL) and BL to AP transport of DMXAA (A and B) and DMXAA-G (C and D) by Caco-2 monolayers. DMXAA or DMXAA-G was loaded on either apical or basolateral side and incubated at 37 °C. At indicated time, samples were collected from receiving side and DMXAA or DMXAA-G was determined by HPLC. Values were mean ± S.D. from 3 to 9 determinations.

± 5.5 μM, and V_{max} of 0.022 ± 0.001 nmol/min. The transport of DMXAA-G (100 μM) from AP to BL, and BL to AP, across Caco-2 cell monolayers was 274- and 10-fold slower than the corresponding values for DMXAA at 100 μM, and that the TEER values of the monolayers did not change significantly ($P > 0.05$) during the experimental period (up to 6 h), even at the highest concentration of DMXAA (500 μM) and DMXAA-G (200 μM). The latter ensured the integrity of the monolayers.

Decrease in the apical pH 5.5–6.5 caused a significant ($P < 0.05$) increase (35%) of P_{app} values for AP to BL flux of DMXAA. However, changing the basolateral pH values did not affect the BL to AP flux of DMXAA-G. The substitution of sodium salts in the transport medium with potassium salts had no significant effect on the transport of DMXAA for either AP to BL or BL to AP. Similarly, this substitution had no significant effect on the AP to BL transport of DMXAA-G. However, it caused a 25% decrease in the BL to AP transport of DMXAA-G, but this change is not statistically significant ($P > 0.05$).

Transport of DMXAA (100 μM) from AP to BL showed some temperature dependency, with a 33.0–42.3% reduction of P_{app} values when the incubation temperature was decreased to 4 °C ($P < 0.05$) compared to those at 37 °C. Similarly, incubation temperature had a significant impact on the transport of DMXAA-G, with the AP to BL and BL to AP transport of DMXAA-G at 4 °C reduced to 50 and 14%, respectively of those values at 37 °C ($P < 0.05$).

The effects of a number of compounds on the transport of DMXAA and DMXAA-G were investigated. Addition of the transport buffer with sodium azide (10 mM) or 2,4-dinitrophenol (1 mM), as well as the absence of glucose in the transport medium did not affect the AP to BL and BL to AP transport of DMXAA. However, both caused a significant ($P < 0.05$) decrease (35 and 37%, respectively) in the BL to AP transport of DMXAA-G, while the AP to BL flux was not significantly influenced. Thalidomide, celecoxib, cyproheptadine, diclofenac, fenoprofen, ketoprofen, ibuprofen, probenecid, verapamil, cimetidine and MK-571 (all at 100 μM) were tested for their ability to alter the AP to BL transport of DMXAA, but none showed any significant effect. Similarly, nifedipine, dexamethasone, cimetidine, probenecid, thalidomide, diclofenac, verapamil, cyclophosphamide, and DMXAA had no significant effect on the DMXAA-G transport from BL to AP. In addition, the hydrolysis products of thalidomide (incubated at 37 °C for 24 h) did not influence DMXAA or DMXAA-G transport. However, MK-571 inhibited the BL to AP efflux of DMXAA-G in a concentration-dependent manner. MK-571 at 50 and 100 μM inhibited the BL to AP efflux of DMXAA-G by 15% ($P > 0.05$) and 40% ($P < 0.05$), respectively, with an estimated K_i value of 130 μM. In ad-

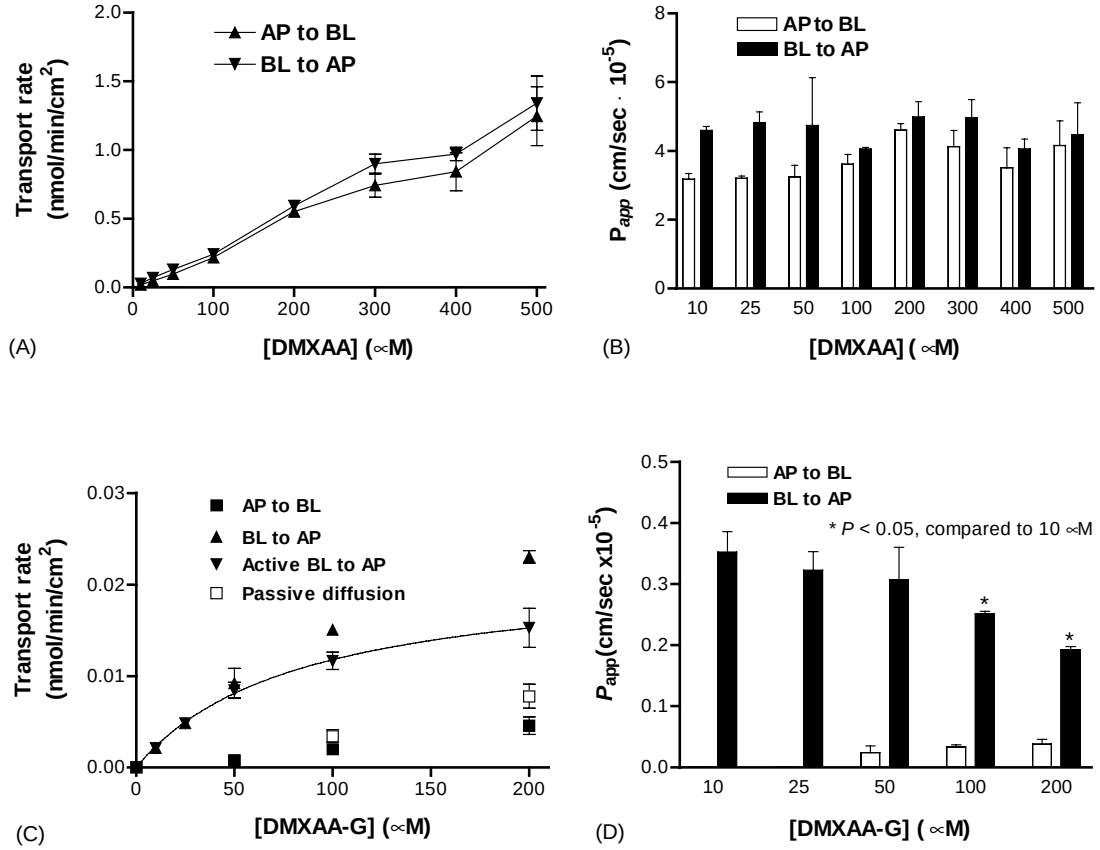

Fig. 7. Effects of concentration on the transport rate and P_{app} of DMXAA (10–500 μM) (A and B) and DMXAA-G (10–200 μM) (C and D) from apical (AP) to basolateral (BL) and BL to AP side. DMXAA or DMXAA-G was loaded on either AP or BL side and incubated at 37 °C. Samples from receiving side were collected and DMXAA or DMXAA-G was determined by HPLC. Data are the mean ± S.D. from 3 to 9 determinations. Curve in part C represents the fit of model with one saturable transport system.

dition, MK-571 (100 μM) caused a significant ($P < 0.05$) increase (36.7%) in the AP to BL transport of DMXAA-G. In the presence of MK-571 (100 μM), the R_{net} at 100 μM DMXAA-G was decreased from 6.7 to 1.9.

4. Discussion

This work presented simple and reliable HPLC methods for the determination of DMXAA and DMXAA-G in transport buffer for Caco-2 monolayers. Validation data indicate all these methods were sensitive and reliable, with acceptable accuracy (85–115% of true values) and precision (intra- and inter-assay CV <15%). The total running time was within 6.8 min, with acceptable separation of the compounds of interest. The LOQ values for DMXAA and its metabolite DMXAA-G were 14.2 and 24 ng/ml, respectively. This high sensitivity was important, as concentrations of DMXAA and its metabolite in the receiving side of the monolayers were very low when using low loading concentrations. Therefore, these HPLC methods are suitable for the analysis of DMXAA and DMXAA-G in transport studies.

DMXAA at concentrations <500 μM appeared to have little direct cytotoxicity against Caco-2 cells, as indicated by its high IC_{50} values (758 and 735 μM) for inhibition of Caco-2 cell growth by either the SRB or ^{3}H-thymidine incorporation assay. The maximum concentration of DMXAA used for our transport studies was 500 μM, which should not be toxic towards the Caco-2 cell monoloayers, especially since the incubation time (up to 6 h) was much shorter than the 96 h employed for the growth inhibition assays. Our results indicated that DMXAA was metabolized by Caco-2 cells to a very limited extent (up to 5.0%). Thus metabolism would not have significant impact on the transport kinetics, and the calculation of a P_{app} over a 1 h exposure would yield a value representative of the transport of DMXAA and DMXAA-G.

DMXAA had a relatively high apparent permeability coefficient >30 × 10^{-6} cm/s in Caco-2 monolayers, similar to that previously reported for many well absorbed drugs [54]. There was a lack of polarized transport for DMXAA and ATP and transporter inhibitors and replacement of extracellular Na^+ with K^+ had no effect on its transport. These findings suggested that the oral bioavailability for DMXAA in humans would be high and that passive transcellular diffusion was the major transport mechanism for DMXAA in Caco-2 monolayers. However, the absorptive (AP to BL) permeability of DMXAA-G (measured at 100 μM, pK_a ≈

3.5) gave a value of 0.38×10^{-6} cm/s, which is comparable to those for relatively hydrophilic compounds such as mannitol (0.48×10^{-6} cm/s in our study and 0.5×10^{-6} cm/s [55], acetylsalicylic acid (2.4×10^{-6} cm/s) and practolol (0.9×10^{-6} cm/s) in Caco-2 monolayers [56]. However, the rate of BL to AP transport of DMXAA-G was 5–12-fold greater than that for AP to BL. MK-571, but not verapamil, nifedipine or probenecid, inhibited the BL to AP transport. In addition, MK-571 increased the AP to BL transport. The BL to AP transport of DMXAA-G was energy and temperature-dependent, but H^+ and Na^+ did not influence this process. All these results indicate that active transport may be involved in the efflux of DMXAA-G, and suggests that MRP1/MRP2, rather than PgP, may be the transporter involved.

In conclusion, the work presented simple and sensitive HPLC methods for the determination of DMXAA and DMXAA-G in HBSS which have been applied to the transport study of both compounds by Caco-2 monolayers. The results indicated that DMXAA was passively transported by intestinal cells, whereas DMXAA-G appears a substrate for MRP1/2. Further studies are needed to study the oral bioavailability of DMXAA in patients and to identify more evidence for the involvement of MRP1/2 in the transport of DMXAA-G.

Acknowledgements

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