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Validation of a liquid chromatography–mass spectrometry method to assess the metabolism of bupropion in rat everted gut sacs

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Abstract

We have developed a rapid, sensitive and selective LC–MS method for the simultaneous assay of bupropion and its metabolite hydroxybupropion during its intestinal absorption, studied with the rat everted gut sac model. The method was validated in the concentration range of 1–15 μM (0.024–3.58 μg/mL) for bupropion and 0.005–1 μM (0.00127–0.25 μg/mL) for hydroxybupropion with 10 μL injected. Bupropion is used as a probe for the activity of the CYP2B6 isoenzyme of the P450 family of enzymes in man. Its major metabolite hydroxybupropion was found in the serosal media of the gut sac showing that the isoenzyme of the 2B group was active in the intestinal mucosa and metabolized bupropion during its passage across the mucosa. The metabolite was also quantified in the mucosal media indicating its ability to cross the apical membrane of the epithelial cells.
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1. Introduction

The high expression of drug metabolizing enzymes in the human small intestine and liver results in extensive first-pass metabolism of many drugs that exhibit low oral bioavailability (<50%). It is now well-recognized that the intestine can play an important role in the metabolism of drugs [1,2] as reported for several different drugs with low bioavailability after oral administration, such as midazolam [3–5], tacrolimus [6,7], the immunosuppressant SDZ-RAD [8], and cyclosporin [9,10]. Evaluating the relative importance of hepatic versus intestinal first pass metabolism remains a challenge [2,11,12]. In man, the major enzymes involved in drug metabolism are members of the cytochrome P450 (CYP) super-family of enzymes of which the isoforms CYP1A, CYP2C9, CYP2D6 and CYP3A4 collectively participate in the oxidation of about 95% of all drugs [13]. Cytochrome P4503A is the most important sub-family in man [14] and these enzymes are also a major site for drug–drug interactions.

The expression of several CYP isoforms has also been reported in the rat small intestine, with 1A1 being the most predominant [15] but also 2B1, 2C6, 2C11, and 2J4, the most recently described [16]. However, the different methods employed to detect the enzymes has led to inconsistencies for some enzymes, such as CYP2C11, reported by certain authors and not detected at all by others in rat intestine [15,17–20]. CYP2D6 is found in the human intestine [21], but its rat homologue CYP2D1 has not been reported to be present in the rat small intestine. However, in a previous study using the rat everted gut sac model incubated with dextromethorphan, we detected the formation of the metabolite dextrorphan, suggesting that CYP2D isoforms are active in the rat intestine [22].

A single CYP isoform is often predominantly responsible for the metabolism of a drug, and some specific substrates have been selected as probes to measure the activity of the CYP3A enzymes in man or their rat homologues such midazolam 1-hydroxylation, testosterone 6β-hydroxylation (CYP3A/1A activity), dextromethorphan *O*-demethylation (CYP2D6/1 activity) [23] and

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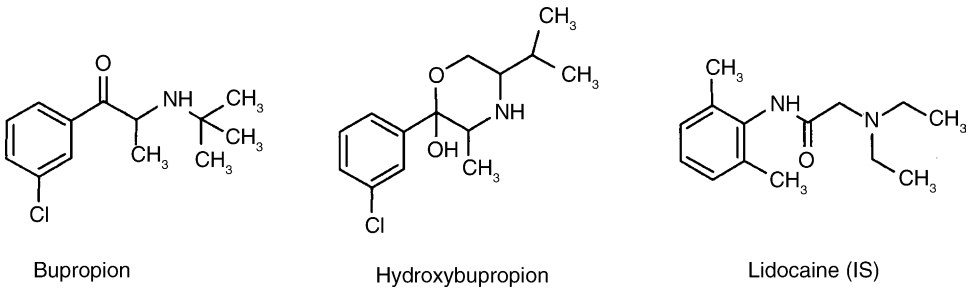

Fig. 1. Chemical structures of bupropion, hydroxybupropion and lidocaine.

bupropion (CYP2B6). However, some of the methodology developed for the simultaneous analysis of CYP enzymes in vitro has the limitation that not all the major drug-metabolizing CYP isoforms can be detected (e.g. CYP2B6) [24]. Whereas the level of expression of CYP2B6 in human liver and its metabolic capabilities are not clear and may have been underestimated, many examples of xenobiotics metabolized by CYP2B6 have been identified [25]. Bupropion hydroxylation has been characterized as a specific probe for CYP2B6 activity [26]. The chemical structure of bupropion and its metabolite are shown in Fig. 1. CYP2B1 is the rat homologue, found to be 75% homologous in protein sequence to the CYP2B6 human enzyme and species differences in metabolic capability are likely to occur [25].

Using the in vitro everted rat gut sac model, we recently demonstrated that metabolism of testosterone and dextromethorphan was occuring during their passage across the gut epithelium with 6β-hydroxytestosterone, androstenedione or dextromethorphan and 3-methoxymorphinan being detected in the serosal media [22,27].

The aim of the current study was to investigate if this model is able to detect the metabolism of bupropion and to develop a LC–MS assay sensitive enough to quantify the small quantities of metabolites formed during the drug absorption through the intestinal enterocytes. Few reports are available in the literature for the simultaneous assay of bupropion and its metabolite hydroxybupropion though a sensitive HPLC-UV method was reported for bupropion alone [28]. Both compounds could be quantified using HPLC-UV methodology after extraction with a good sensitivity for bupropion (LQ = 5 ng/mL) but to a lesser extent for the metabolite (LQ = 100 ng/mL) [29].

A more sensitive semi-automated LC–MS/MS method was recently developed for the assay of bupropion from human, rat or mouse plasma after ethyl acetate extraction with a LQ = 0.25 ng/mL and 1.25 ng/mL, respectively, for bupropion and its metabolite [30]. We have developed a new LC–MS method after an extraction procedure without an evaporation step. The method was validated for the quantification of bupropion and its metabolite in the TC 199 medium used to ensure a good viability of the everted gut sacs.

2. Experimental

2.1. Chemicals

Bupropion, lidocaine, tissue culture medium TC 199 (10× concentrated with Earle's salts), and glutamine were purchased from Sigma–Aldrich Chimie (St. Quentin Fallavier, France); hydroxybupropion was provided by Gentest (San Jose, USA), and formic acid was from J.T. Baker (Phillipsburg, USA). Diethylether and methanol (SDS, France) were of HPLC grade and used without further purification. Ultrapure water was obtained using a Millipore Simplicity 185 apparatus.

2.2. Gut sac preparation and incubation

Male Sprague–Dawley rats (220–240 g weight, DEPRE, Saint Doulchard, France) were used in our experiments. The medium was TC 199 (with Earle's salts), pH 7.3, and gassed by bubbling at 37 °C with 95% O_2/5% CO_2. After an overnight fast, the rats were humanely sacrificed by a qualified person, and the entire small intestine quickly excised and flushed through several times with NaCl solution (0.9%, w/v) at room temperature. The intestine was immediately placed in warm (37 °C), oxygenated TC 199 medium and then gently everted over a glass rod (2.5 mm diameter). One end of the intestine was clamped and the whole length of the intestine was filled with fresh oxygenated medium and sealed with a second clamp and the resulting large gut sac divided into sacs of approximately 2.5 cm in length using braided silk sutures. For each experiment, 12–15 sacs were prepared, starting from the end of the duodenum, to ensure that sacs were from the upper/mid jejunum where metabolic activity is maximal. Each experiment was carried out using the intestine from one rat with each sac being placed in an Erlenmeyer flask (50 mL) containing a solution of bupropion (100 μM) in TC 199 medium pregassed with 95% O_2/5% CO_2 at 37 °C. Flasks were stoppered with gas-tight silicon bungs and incubated at 37 °C in a shaking water bath (60 cycles/min). At the appropriate time points, sacs were removed, washed three times in saline and blotted dry. The sacs were cut open and the serosal fluid drained into small tubes. Each sac was weighed before and after serosal fluid collection to calculate accurately the volume inside the sac. The sacs were then digested individually in 25 mL of 1 M NaOH at 37 °C for 2 h. The protein content of the digest was determined spectrophotometrically using the method described by Peterson [31] with bovine serum albumin as standard. Samples of the medium and serosal fluid were kept for extraction for the LC–MS analysis. From the sac contents volume, the quantity of bupropion and hydroxybupropion was calculated and the appearance on the serosal side of the epithelium expressed as nanomoles or picomoles per mg of tissue protein. To compare the uptake of the bupropion with other drugs studied with the

Table 1
Retention times of the IS, bupropion and hydroxybupropion and the related molecular ion $[M+H]^+$ selected in the SIR function

	t_r (min)	m/z $[M+H]^+$
Lidocaine (IS)	3.07	235.14
Bupropion	7.80	240.05
Hydroxybupropion	6.07	256.05

everted gut sac, the apparent permeability (P_{app}) was calculated as previously described [32].

2.3. LC–MS conditions

The LC–MS system consisted of an Alliance 2695 separation module interfaced to a ZQ mass spectrometer equipped with an electrospray ionisation source (Waters, St. Quentin, France). A Waters Sentry C_{18} (2.1 mm × 10 mm, 3.5 μm) guard column and a Waters Symmetry C_{18} column (2.1 mm × 150 mm, 5 μm) were used for separation. Analyses were run in positive mode with the capillary and cone voltages set to 3 kV and 20 V, the temperature of the heated capillary at 250 °C and the nitrogen nebulizing gas flow set at 350 L/h and the cone gas flow at 150 L/h. The mobile phase was water/formic acid 1%/methanol (65:10:25, v/v/v) used at a flow rate of 0.2 mL/min for a run time of 10 min. Other acidic additives, such as acetic acid or trifluoacetic acid tested in the mobile phase decreased the sensitivity. Because of the similarity between the structures of bupropion and lidocaine, the latter (Fig. 1) was used as internal standard (IS) at a concentration of 0.125 μM for all the quantifications. The apparatus was managed with a Masslynx software (Micromass, version 3.5). Analyses were run in the selected ion recording mode (SIR) by selecting the molecular ion $[M+H]^+$ of each compound (Table 1).

2.4. Sample extraction and recovery

The same lidocaine IS stock solution (5 mM) was used in the preparation of samples for quantification and stored at −20 °C. The IS was then diluted to have a concentration of 5 μM and 25 μl was added to 0.25 mL of serosal fluid or 0.05 mL of mucosal media, and the final volume was adjusted to 1 mL with TC199 media for all samples before extraction. After addition of 1 M NaOH (20 μl), the mixture was stirred and 1 mL of diethylether was added, vigorously shaken and briefly centrifuged (2 min, 3000 × g). The organic phase was removed and 1 mL of formic acid (0.5%, v/v) in water was added to the organic layer and after agitation and centrifugation (2 min, 3000 × g), the aqueous phase (10 μL) was injected for LC–MS analysis.

Recovery studies were performed by extracting the target compound from TC 199 medium. One millilitre of TC 199 medium was spiked with appropriate amounts of bupropion and hydroxybupropion and they were extracted by the procedure reported above. Relative recovery experiments were performed by comparing the chromatogram area obtained after extraction with the area obtained with the target compound without extraction. Relative recoveries were carried out in triplicate for different concentrations within the calibration range.

2.5. Method validation

Stock solutions of bupropion (5 mM), hydroxybupropion (3.9 mM) and lidocaine (5 mM) were prepared and kept at −20 °C before being suitably diluted to prepare calibration solutions and quality control (QC) samples. Standards were prepared by serial dilution with TC199 of the initial bupropion and hydroxybupropion stocks to obtain the following concentrations: 0.005; 0.01; 0.025; 0.05; 0.1; 0.25; 0.5; 0.75 and 1 μM for hydroxybupropion and 0.1; 0.25; 0.5; 0.75; 1.25; 5; 7.5; 8; 12.5 and 15 μM for bupropion. Within-day precision was determined using QC samples prepared in replicates (n = 5) at four concentrations (QC = 0.25; 1; 7.5 and 12.5 μM) for bupropion and three different concentrations (QC = 0.01; 0.1; 0.75 μM) for hydroxybupropion. This operation was repeated by preparing and analysing five fresh independent replicates the next day and 2 days later again with three fresh replicates to assess between-day precision and accuracy.

The stability of the stock solutions was studied by the comparison of related bupropion and hydroxybupropion areas in chromatograms obtained with fresh QC solutions and QC prepared from stock solutions stored at −20 °C. The same areas (CV < 9%) were obtained for all QC samples after 4 months and 7 months at −20 °C, respectively, for bupropion and the metabolite indicating the absence of degradation during the storage. The IS stock solution was remade after 2 months at −20 °C and we did not detect any modification in the chromatogram area after this storage period.

TC 199 medium was tested after extraction (blank without analytes) for any chromatographic matrix effects and no peak was detected on the corresponding chromatogram. Analysis of TC 199 medium spiked with the IS gave only its characteristic peak at 3.2 min and no significant variation of the chromatogram was observed when extraction was performed from TC 199 medium containing the IS and a gut sac without analytes.

3. Results

3.1. Chromatographic separation and detection

Analyses were run in the selected ion recording mode by selecting the molecular ions $[M+H]^+$ at m/z = 250.05, m/z = 256.05 and m/z = 235.14, respectively for bupropion, hydroxybupropion and lidocaïne. Calibration graphs were constructed by plotting peak area ratios versus analyte concentrations using a least-square linear regression model with a good linearity in the concentration range tested: 0.1–15 μM bupropion and 0.005–1 μM for hydroxybupropion as indicated by the regression data (r > 0.997) for both compounds.

The back extraction of bupropion and its metabolite after treatment of the sample by diethylether is a convenient method and gave an extraction recovery over 80% (Table 2) with good linearity and repeatability without any evaporation step.

Table 2
Extraction recoveries from TC 199 medium

	Concentration (μM)	Extraction recoveries (%) Mean ± S.D. (n = 5)
Lidocaïne	0.125	90.2 ± 8.2
Bupropion	0.25	82.1 ± 11.8
	1.00	90.8 ± 1.7
	7.50	85.5 ± 10.2
	12.5	96.6 ± 4.4
Hydroxybupropion	0.01	85.5 ± 8.8
	0.10	96.3 ± 5.5
	0.75	102.7 ± 9.0

3.2. Precision and accuracy

Percent relative standard deviation (R.S.D., %) was calculated as an estimation of precision. Accuracy (relative bias) was expressed as a percentage and calculated by the agreement between the intra-day measured values and the nominal concentration of the spiked standard samples. As can be seen in Table 3, the overall precision for within-day analyses ranged from 2.7 to 12.6% while the accuracy was from 16.2 to 11.2%. The between-day assay precision ranged from 7.0 to 15.7% while the accuracy was from 1.3 to 16.4%. Thus, the method had good precision and accuracy given the complexity of the matrix for the quantification of bupropion and hydroxybupropion in TC 199.

Table 3
Validation of bupropion and hydroxybupropion assays in TC199 medium

	Linearity			
	Slope (n = 5)	r	CV (%)	
Bupropion	0.48 ± 0.024	0.997–0.999	5.0	
Hydroxybupropion	0.34 ± 0.035	0.998–0.999	8.9	
Bupropion (μM)	0.25	1.00	7.50	12.50
Within-day precision				
Mean (n = 5)	0.21	1.01	7.61	12.10
CV (%)	11.0	2.7	10.8	6.3
Accuracy (% bias)	−16.2	1.2	1.4	−3.4
Inter-day precision				
Mean (n = 13)	0.22	1.10	7.70	12.49
CV (%)	15.7	10.1	9.4	7.0
Accuracy (% bias)	−11.3	8.2	2.7	−0.08
Hydroxybupropion (μM)	0.010	0.100	0.75	
Within-day precision				
Mean concentration (n = 5)	0.011	0.093	0.76	
CV (%)	12.6	4.4	11.0	
Accuracy (% bias)	11.2	−7.2	1.3	
Inter-day precision				
Mean concentration (n = 13)	0.011	0.096	0.75	
CV (%)	11.9	7.5	10.6	
Accuracy (% bias)	16.4	−3.4	0.3	

3.3. Sensitivity

A signal-to-noise ratio greater than 10 (S/N > 10) is usually used to set the quantification limits (LOQ) and this ratio was obtained when 5 μL of a 0.005 μM solution (25 fmoles, 5.9 pg injected) was injected for bupropion and 10 μl of a 0.01 μM solution (10 nmoles, 25 pg injected) for hydroxybupropion. As shown in Table 2, the precision and accuracy at a metabolite concentration of 0.01 μM did not exceed 16% and met the required criteria to be considered as the LOQ while the experimental concentrations of hydroxybupropion measured in serosal and mucosal samples were all above 0.011 μM.

As the amount of bupropion is high compared to the metabolite (>4.1 μM in all serosal and mucosal samples), it was not necessary for the calibration curve to descend to the LOQ and a concentration range from 0.1 to 15 μM was chosen, where the lowest point is considerably higher than the LOQ.

However, to have an idea of the performance of the method, we determined the concentration giving a signal-to-noise ≥10 to evaluate the LOQ for bupropion (n = 5) and found a concentration of 0.005 μM. As all our experimental measurements were at higher concentrations it was not necessary to determine the precision and accuracy as for QC samples. From the concentrations measured for the standard samples used for the signal-to-noise analysis (made the same day as a calibration curve), we deduced the intra-day precision (n = 5, CV (%) = 23.5) and accuracy (n = 5, CV (%) = 16.3).

3.4. Bupropion metabolism in the rat everted gut sac

A comparison with the typical chromatogram of the standard compounds (Fig. 2A) showed that bupropion (Fig. 2B.3) and hydroxybupropion (Fig. 2B.2) were both detected in the serosal medium as can be seen in the HPLC–MS SIR traces of the serosal content of the everted gut sac, after incubation in a 100 μM bupropion solution in TC 199 medium for 15 min; and extracted as described in Section 2. Figs. 3 and 4A show that the amount of bupropion and hydroxybupropion increased in the serosal content as a function of incubation time. After 30 min incubation, the metabolite was also detected and quantified (Fig. 2C.2) on the mucosal side of the gut sac and increased with time (Fig. 4B).

4. Discussion

The objective of this study was to develop an analytical method that would enable direct quantitative and qualitative studies of metabolism probes during intestinal absorption using an in vitro system. We previously showed that methadone [33], testosterone [27] and dextromethorphan [22], respectively, used as CYP 3A4/1 and 2D6/1 probes could be metabolized during their absorption by the intestine.

We present here the validation of an LC–MS method developed for the quantification of bupropion intestinal absorption and metabolism (hydroxybupropion formation) using the rat everted gut sac model. The small quantities of material available from in vitro methods meant that the analytical system

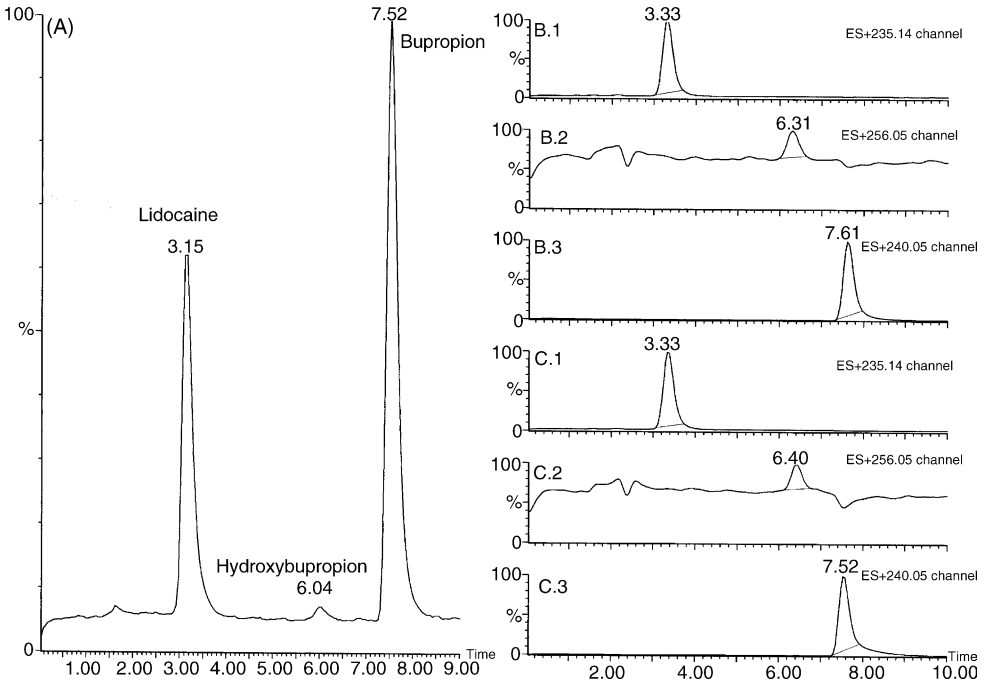

Fig. 2. (A) Selected ion recording (SIR) chromatogram of standard compounds lidocaine (IS, 0.125 μM), hydroxybupropion (0.01 μM) and bupropion (0.25 μM). (B.1–3) Quantification SIR chromatographic traces of a serosal medium and (C.1–3) a mucosal medium after incubation of the gut sacs (respectively, 15 and 30 min) with bupropion at 100 μM. 1, 2, and 3 correspond, respectively, to the related characteristic ion channels used for the quantification of the IS (m/z = 235.14), hydroxybupropion (m/z = 256.05) and bupropion (m/z = 240.05). The concentrations measured were 0.125 μM for the IS (B.1 and C.1); 0.019 μM (B.2) and 0.013 μM (C.2) for hydroxybupropion; 6.92 μM (B.3) and 11.3 μM (C.3) for bupropion.

needed to be very sensitive, accurate, and discriminatory as well as capable of analysing the metabolites in the presence of tissue culture medium, which contains a wide variety of chemicals and salts. Thus, it was essential to clean samples by a preliminary extraction procedure in order to avoid signal suppression and improve the sensibility. Whereas extraction recoveries are often not detailed in the literature data, it is crucial to have an idea of the extraction efficiency.

Ethyl acetate, previously used for extraction from plasma with a semi-automated 96-well plate liquid–liquid method [30] gave a satisfactory extraction yield in TC199 of up to 70% for both compounds. Furthermore, it is more convenient to recover the upper layer in a manual extraction procedure using diethylether. As the evaporation step could be critical if bupropion (boiling point 52°) remained partially in its basic form in the extract, we opted for an extraction strategy using diethylether without evaporation as reported in Section 2 and the method was validated for absorption and metabolism studies of bupropion in the rat everted gut sac.

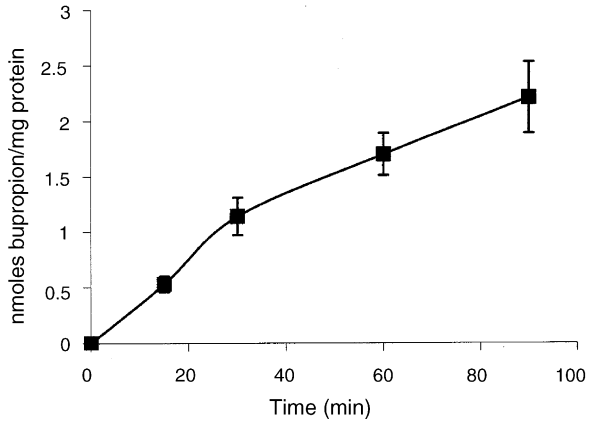

Fig. 3. Appearence of bupropion in the serosal contents (mean ± S.D., n = 6).

As shown in Fig. 3, bupropion is absorbed across the intestinal mucosa and we have calculated the apparent permeability (P_{app}) of bupropion. The value was $5.68 \pm 0.46 \times 10^{-5}$ cm/s which was in the same order of magnitude as those values obtained for the P_{app} of testosterone [27] and dextromethorphan [22], two other lipophilic compounds studied with the rat everted gut sac model. These drugs were metabolized during their passage across the epithelial cells and in the present study hydroxybupropion was recovered in the serosal medium inside the sac showing the catalytic activity of CYP2B enzymes in the rat intestinal mucosa. Bupropion provided another example of a drug usually used as a specific probe (CYP 2B6/1) and which was metabolized in the rat intestinal tissue. As is the case with many drugs, the pharmacokinetic and clinical significance of the intestinal metabolism is not clear [11,12], and unravelling the roles of intestinal versus hepatic metabolism in humans is technically and ethically difficult. In man buproprion is extensively metabolized (≈99%) [34].

After being formed in the enterocytes, drug metabolites could cross the basolateral membranes of the cells into the serosal compartment or cross the apical membrane to the mucosal side. Fig. 4 shows that the buproprion metabolite was detected in the mucosal medium and, in fact, the rate of transport (nmoles/mg protein) across the apical cell membrane was substantially higher than in the serosal direction. While the

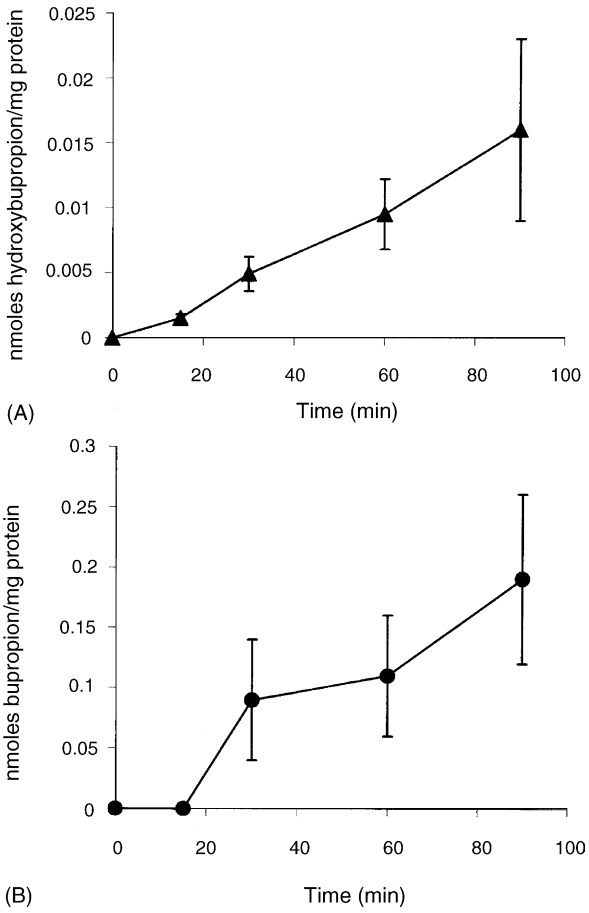

Fig. 4. Appearence of hydroxybupropion in the serosal contents (A) and on the mucosal side (B) of the gut sac (mean ± S.D., $n = 6$).

presence of metabolites in the mucosal medium could be due to simple diffusion, it may also be indicative of the activity of the well-documented efflux transport mechanisms in the apical membrane. P-glycoprotein is the most studied of the efflux transporters and has a very broad substrate specificity, although other transporters, such as MRP2 (multidrug resistance protein 2) and BCRP (breast cancer resistance protein) may also be important in xenobiotic efflux from the enterocytes. The presence of metabolites in both the serosal and mucosal compartments was also detected with other substrates whose metabolism was previously studied with the everted gut sac system, for example, dextromethorphan [22], which has been reported to be a P-glycoprotein substrate [35]. It has not been reported whether bupropion is a transporter substrate, through its structure and lipophilicy might predispose it to be so. The interplay between the metabolic enzymes and efflux drug transporters is currently a subject of considerable interest and debate [36] and the everted gut sac model could be very valuable for evaluating this interplay by the use of specific inhibitors of the respective systems. Such studies are now in progress in our laboratory.

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