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

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Abstract

A rapid, sensitive and selective liquid chromatography–mass spectrometry (LC–MS) method was developed for the simultaneous assay of dextromethorphan and its metabolites in tissue culture medium and its intestinal metabolism studied with the rat everted gut sac model. The method was validated in the concentration range of 0.1–2.5 μM (27.1 ng/mL–0.677 μg/mL) for dextromethorphan and 0.005–0.5 μM for dextrorphan and 3-methoxymorphinan (1.28 ng/mL–0.128 μg/mL) and 3-hydroxymorphinan (1.22 ng/mL–0.122 μg/mL). The limits of quantification (LOQ) were 0.0025 μM (12.5 fmoles, 3.4 pg, 5 μL injected) for dextromethorphan; 0.0025 μM for dextrorphan, 3-methoxymorphinan (24.9 fmoles, 6.4 pg injected), and 3-hydroxymorphinan (25.1 fmoles, 6.1 pg injected) with 10 μL injected. The detection of dextrorphan and 3-methoxymorphinan showed that both the P450 isoforms CYP3A and 2D were active in the intestinal mucosa and metabolised dextromethorphan during its passage across the mucosa.

Keywords: Dextromethorphan; LC–MS; Intestine; Metabolism; Cytochrome P450

1. Introduction

The intestine can play an important role in the biotransformation of xenobiotics which undergo first-pass metabolism in the gastro-intestinal tract, and the metabolism of drugs when passing through the intestinal enterocytes has been a focus of attention in recent years. 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 [1]. The CYP3A is the most important sub-family in man [2] and these enzymes are also a major site for drug–drug interactions. The rat small intestine is reported to express several of the CYP isoforms, including 1A1, 2B1, 2C6, 2C11, and 3A1, with the 1A1 being the most predominant [3]. An additional isoform, 2J4, has also recently been described [4]. However, there are considerable discrepancies in the literature, with some of the enzymes, e.g. CYP2C11, reported by certain authors and not detected at all by others [3,5–8]. This may be as a consequence of the different methods employed which often detect the presence of the appropriate messenger RNA (Northern blotting) or enzyme protein using antibodies (Western blotting) but not actual enzyme activity. While CYP2D6 is found in the human intestine [9], its rat homologue CYP2D1 has been found in the liver and the brain of the rat [10–12], but there are no reports of it being present in the rat small intestine.

Although CYP isoforms show overlapping substrate specificity, a single CYP isoform is often predominantly responsible for the metabolism of a drug. For example, midazolam 1-hydroxylation and testosterone 6β-hydroxylation (6β-hydroxytestosterone (6β-OHT) formation) are widely

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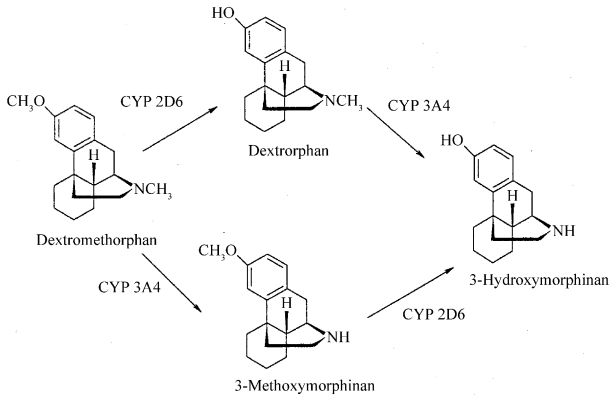

Fig. 1. Chemical structures and metabolic pathways of dextromethorphan in man.

used as probes to measure the activity of the CYP3A enzymes. Dextromethorphan *O*-demethylation to dextrorphan is used specifically to detect human CYP2D6 activity both in vitro and in vivo [13]. In fact, dextromethorphan undergoes parallel *O*-demethylation to dextrorphan and *N*-demethylation to 3-methoxymorphinan, while a didemethylated metabolite 3-hydroxymorphinan is formed as a secondary metabolite (Fig. 1). While *O*-demethylation activity is well established as an index reaction of CYP2D6/1, the reaction may be mediated in man by at least two different enzymes in vitro and in vivo as indicated by a biphasic Eadie–Hofstee plot [14]. Nevertheless, as this pathway was shown to be primarily CYP2D6 dependent in man it was therefore considered to be useful as an index reaction for CYP2D6/1. Dextromethorphan *N*-demethylation (3-methoxymorphinan) has been proposed to reflect CYP3A4 activity in man but there are also suggestions that the reaction may be a marker to monitor simultaneously CYP3A and CYP2D6 activity. This is based on the observation that purified recombinant human CYP2D6 produced significant amounts of *N*-demethylated methoxymorphinan and hydroxymorphinan in addition to dextrorphan [13].

The role of the first pass metabolism in the small intestine [15] has been reported in recent years for several different drugs with low bioavailability after oral administration such as midazolam [16–18], tacrolimus [19,20], the immunosuppressant SDZ-RAD [21], and cyclosporin [22,23]. Consequently, it is very important to study the behaviour of drugs passing through the intestinal enterocytes. We recently demonstrated that testosterone 6β-hydroxylation activity is present in rat intestine [24]. We used an in vitro everted rat gut sac system which has proved very valuable for studying various aspects of drug absorption [25–29]. This model was successful for studying simultaneously the absorption and metabolism of testosterone by the epithelium with 6β-hydroxytestosterone and androstenedione being detected in serosal media [24], clearly demonstrating that metabolism, notably by a CYP3A isoform, was occurring during the passage across the gut epithelium. The aim of the current study was to investigate if this model is able to detect the metabolism of other CYP isoenzymes substrates, and our results with testosterone prompted us to extend this work to dextromethorphan, another well characterised probe of P450 metabolism.

As only small quantities of metabolites are formed in the in vitro gut sac model, very sensitive assays are required. Among the LC–MS/MS [30–33] or LC fluorescence [34,13] methods described in the literature to quantify dextromethorphan and/or metabolites with high sensitivity, none were reported to quantify all the compounds together. We report herein the development of a sensitive liquid chromatography–mass spectrometry (LC–MS) assay for dextromethorphan and three of its metabolites: dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan. Dextromethorphan and its metabolites could be accurately quantified on both sides of the rat everted gut sac model as all these compounds could be satisfactorily recovered from the complex TC 199 tissue culture medium and the method was validated in this medium.

2. Experimental

2.1. Chemicals

Dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan, codeine, tissue culture medium TC 199 (10× concentrated with Earle's salts), and glutamine were purchased from Sigma–Aldrich Chimie (St Quentin Fallavier, France); formic acid and acetic acid were from J.T. Baker (Phillipsburg, USA). Dichloromethane, hexane 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 approximatively 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 dextromethorphan (100 μM) in TC

Table 1
Extraction recoveries from TC 199 tissue culture medium

Compounds	Extraction recoveries (% ± SD)*		
Dextromethorphan	0.25 μM	0.8 μM	2 μM
	99.5 ± 11.1	85.1 ± 1.9	96.0 ± 4.4
Dextrorphan	0.0075 μM	0.04 μM	0.25 μM
	99.8 ± 12.6	99.8 ± 8.2	90.0 ± 5.4
3-Methoxymorphinan	100.5 ± 7.5	96.4 ± 9.1	99.8 ± 10.0
3-Hydroxymorphinan	74.4 ± 15.6	86.9 ± 8.9	85.6 ± 9.3

* Each recovery is the mean of six independent extraction experiments.

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 [35] with bovine serum albumin as standard. Samples of the medium and serosal fluid were kept for extraction before the LC–MS analysis. From the sac contents volume the quantity of each compound was calculated and the appearance expressed as nanomoles or picomoles per mg of tissue protein. To compare the uptake of the dextromethorphan with other drugs studied with the everted gut sac, the apparent permeability (P_{app}) was calculated as previously described [25].

2.3. Extraction and recovery

Recovery studies were performed by extracting the target compound from TC 199 medium (1 mL) in three replicates. 1 mL of TC 199 medium was spiked with the appropriate quantity of dextromethorphan and metabolites and they were extracted by the addition of 1 mL of dichloromethane/hexane (1/1, v/v) and 5 μL of 10% acetic acid. Samples were vigorously shaken and the organic phase was removed. The aqueous phase was extracted a second time with 1 mL of dichloromethane/1-butanol (2/1, v/v). After agitation, the second organic layer was added to the first one and they were both evaporated under nitrogen at 40 °C and the residue was then dissolved in water (1 mL) before analysis. The recovery was determined by comparing the chromatogram area obtained after extraction with the area obtained with the target compound prepared in methanol but without extraction. Recoveries were carried out in triplicate for different concentrations within the calibration range. The results are shown in Table 1.

2.4. 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) containing the same packing materials were used for the chromatographic separations as dextromethorphan analysis had been previously reported using C_8 (Symmetry [30,31,36]) or C_{18} (Phenomenex [32]) as column packing materials. Analyses were run in positive mode with the capillary and cone voltages set to 1 KV and 40 V, the temperature of the heated capillary at 300 °C and the nitrogen nebulizing gas flow set at 300 L/h. The mobile phase consisted of a mixture of water (A), formic acid 1% (B), and methanol (C) following the gradient program reported in Table 2, with a flow rate of 0.2 mL/min for a run time of 20 min. Methanol/water/formic acid had given good sensitivity for dextromethorphan analysis when used as an eluent by other researchers [30,31,36]. We tested 100 mM ammonium formate as a solvent (10%) with methanol and water but the sensitivity decreased and peaks were tailed. The concentration of codeine used as Internal Standard (IS) was 0.5 μM for all the quantifications. The apparatus was managed with a Masslynx software (Micromass, version 3.5). Analyses were run in the SIR mode (Selected Ion

Table 2
Gradient program for the LC analysis

Time (min)	% A, water	% B, formic acid 1%	% C, methanol	Curve type
0	80	10	10	Convex*
4	50	10	40	Convex*
8	25	10	65	Linear
10	15	10	75	Linear
11	80	10	10	Linear
20	80	10	10	Linear

* Solvent composition change immediately to set the following composition.

Table 3
The MS method for the detection of the dextromethorphan and metabolites

Compounds	Retention times (min)	SIR 1 m/z $[M+H]^+$	SIR 2 m/z $[M+H]^+$
Codeine (IS)	3.98	300.07	300.07
Dextromethorphan	10.19		272.18
Dextrorphan	7.31	258.17	
3-Hydroxymorphinan	7.59		244.10
3-Methoxymorphinan	10.92	258.17	

Recording) by selecting the molecular ion $[M+H]^+$ of each compound. Two SIR functions were used to detect dextromethorphan and 3-hydroxymorphinan, on one hand, and dextrorphan and 3-methoxymorphinan on the other (Table 3).

2.5. Sample preparation

The same codeine Internal Standard stock solution (3.7 mM) was used in the preparation of samples for quantification, with two solutions prepared from the stock solution to give concentrations of 10 and 1 μM. For the analysis of metabolites in the serosal fluid, 0.2 mL of serosal fluid was added to 0.75 mL of TC 199 medium and 0.05 mL of codeine (1 μM) added as Internal Standard. The mixture was then acidified (5 μL of 10% acetic acid) before extraction as reported above. After agitation, and evaporation to dryness under nitrogen, the residue was dissolved in 0.1 mL of water for the quantification of the metabolites by LC–MS with a final IS concentration of 0.5 μM. For the quantification of dextromethorphan in the serosal fluid, 0.05 mL of the IS (10 μM) was added to 0.025 mL of serosal fluid and diluted with 0.925 mL of TC 199 medium. After acidification, extraction and evaporation of the organic layer, the residue was dissolved this time in 1 mL of water, and consequently the final IS concentration was also 0.5 μM. For the mucosal fluid, 0.025 mL was removed, diluted with 0.925 mL of TC 199 and 0.05 mL of the IS (10 μM) was added. Samples were then extracted as described for serosal fluid, and after evaporation the residue was dissolved in 1 mL of water, giving a final concentration of 0.5 μM for the IS.

2.6. Method validation

TC 199 medium was tested after extraction for any matrix effects and no significant matrix variation was observed with six replicates of TC 199 medium blanks without analytes but spiked with the IS. The selectivity and retention time (RT) of each analyte was determined after injection of each compound individually (see Table 4). A stock solution of each standard (in water) was diluted in water to produce the mixture of standard compounds at a concentration of 0.1 μM for each one. As the structure of metabolites was very similar, with a single chromatogram we did not obtain a good separation for the pairs dextromethorphan (RT = 10.19)/3-methoxymorphinan (RT = 10.92) and dextrorphan (RT = 7.31)/3-hydroxymorphinan (RT = 7.59). Therefore, we used two MS SIR functions to produce two independent chromatograms from which we could quantify dextromethorphan and 3-hydroxymorphinan from one SIR trace and dextrorphan and 3-methoxymorphinan from the second SIR chromatogram.

The methods were validated following FDA guidelines and recent recommendations for analytical validations in biological matrices [37,38]. Within-day precision was determined by preparing five replicates on the same day at three concentrations (0.25, 0.8, 2 μM) for dextromethorphan and three different concentrations (0.0075, 0.04, 0.25 μM) for the metabolites. This operation was repeated by preparing and analysing fresh independent replicates for three days to assess between-day precision and accuracy.

3. Results

3.1. Chromatographic separation and detection

Analyses were run in the selected ion recording (SIR) mode by selecting the molecular ions $[M+H]^+$ for each compound. The SIR chromatograms following the analysis of standard compounds (dextromethorphan, metabolites and IS, all at 0.1 μM) spiked in TC 199 and extracted as described in Section 2 are presented in Fig. 2. The $[M+H]^+$ ions were selected for subsequent analysis at m/z = 272.18, 244.10 and 300.07 respectively for dextromethorphan, 3-hydroxymorphinan and the internal standard, and 258.17 for both dextrorphan and 3-methoxymorphinan.

Table 4
Repeatability of the calibration curve after extraction in TC 199 culture medium

Compounds	Slope (mean)	CV (%)	R^2
Dextromethorphan	3.40 ± 0.096 (n = 4)	2.8	0.992–0.999
3-Methoxymorphinan	4.67 ± 0.58 (n = 4)	12.4	0.992–0.999
3-Hydroxymorphinan	2.27 ± 0.267 (n = 5)	11.7	0.992–0.998
Dextrorphan	5.54 ± 0.65 (n = 4)	11.8	0.995–0.998

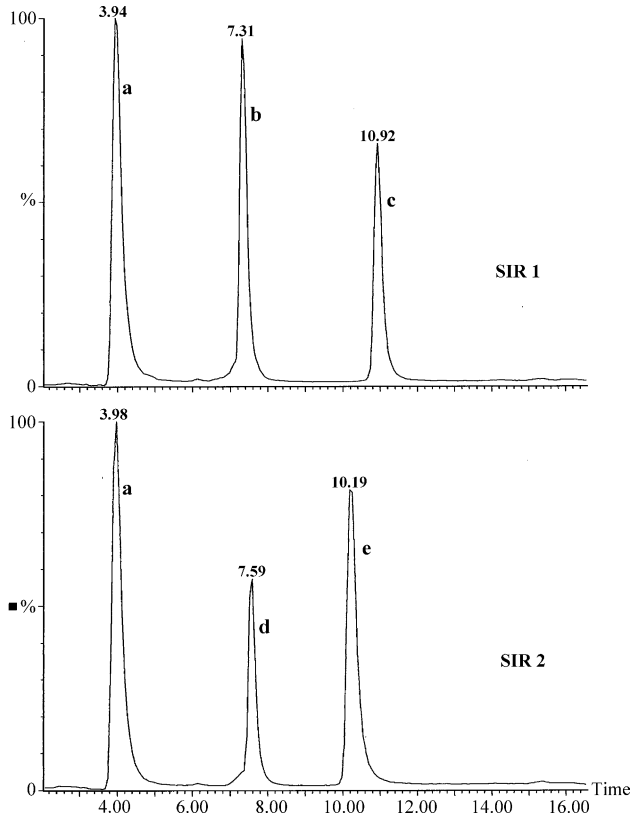

Fig. 2. Selected ion recording chromatograms of: (a) IS (codeine); (b) dextrorphan; and (c) 3-methoxymorphinan (SIR 1); (d) 3-hydroxymorphinan; and (e) dextromethorphan (SIR 2) obtained with standard solutions (0.1 μM) prepared in TC 199 culture medium and then extracted as described in Section 2.

Calibration graphs were constructed by plotting peak area ratios versus analyte concentrations using a least-square linear regression model. The linearity was very good for all analytes in the concentration range tested: 0.1–2.5 μM for dextromethorphan and 0.005–0.5 μM for the metabolites as indicated by the regression data (Table 4), and the variation on the slope was acceptable after liquid extraction (CV < 12.5%).

3.2. Precision and accuracy

Relative standard deviation (RSD%) was calculated as an estimation of precision. Accuracy (relative error) was expressed as a percentage and calculated by the agreement between the measured values and the nominal concentration of the spiked standard samples. As can be seen in Table 5, for within-day analyses the overall precision ranged from 6.9 to 17.8% while the accuracy was from −15.28 to 9.96%. The overall precision for between-day assays ranged from 6.6 to 17.5% while the accuracy was from −7.4 to 10.4%. These values were considered satisfactory, given the complexity of the matrix, and these results demonstrated that the method had good precision and accuracy.

Table 5
Validation of dextromethorphan and metabolites assay in TC 199 culture medium

Dextromethorphan (μM)	0.25	0.80	2.00
Intra-day precision			
Mean (n = 6)	0.25	0.84	1.93
CV%	12.14	6.87	7.79
Accuracy (% bias)	1.60	5.27	−3.65
Inter-day precision			
Mean (n = 12)	0.24	0.82	2.12
CV%	16.24	6.57	14.80
Accuracy (% bias)	−2.03	2.73	6.00
3-Methoxymorphinan (μM)	0.0075	0.040	0.25
Intra-day precision			
Mean (n = 6)	0.0082	0.040	0.22
CV%	10.35	7.68	9.13
Accuracy (% bias)	9.96	−1.25	−13.5
Inter-day precision			
Mean (n = 12)	0.0082	0.037	0.23
CV%	10.61	11.63	16.72
Accuracy (% bias)	9.94	−6.63	−6.72
Dextrorphan (μM)	0.0075	0.040	0.25
Intra-day precision			
Mean (n = 5)	0.0080	0.037	0.23
CV%	17.79	12.21	13.93
Accuracy (% bias)	−15.28	−7.29	−10.16
Inter-day precision			
Mean (n = 11)	0.0077	0.037	0.23
CV%	12.76	13.08	16.04
Accuracy (% bias)	2.97	−7.39	−6.07
3-Hydroxymorphinan (μM)	0.0075	0.040	0.25
Intra-day precision			
Mean (n = 5)	0.0075	0.043	0.24
CV%	14.40	12.30	10.52
Accuracy (% bias)	0.29	7.90	−2.27
Inter-day precision			
Mean (n = 11)	0.0083	0.040	0.24
CV%	17.65	14.04	9.93
Accuracy (% bias)	10.45	1.55	−2.80

3.3. Sensitivity

The limits of quantification (LOQ) were determined with the Signal to Noise ratio greater than 10 (S/N > 10). They were 0.0025 μM (12.5 fmoles, 3.4 pg injected) for dextromethorphan with an injection volume of 5 μL and 0.0025 μM for the metabolites: dextrorphan (24.9 fmoles, 6.4 pg injected), 3-methoxymorphinan (24.9 fmoles, 6.4 pg injected) and 3-hydroxymorphinan (25.1 fmoles, 6.1 pg injected) with an injection volume of 10 μL.

3.4. Dextromethorphan metabolism in the rat everted gut sac

Fig. 3 shows the HPLC–MS SIR traces of the serosal content of the everted gut sac, removed after incubation

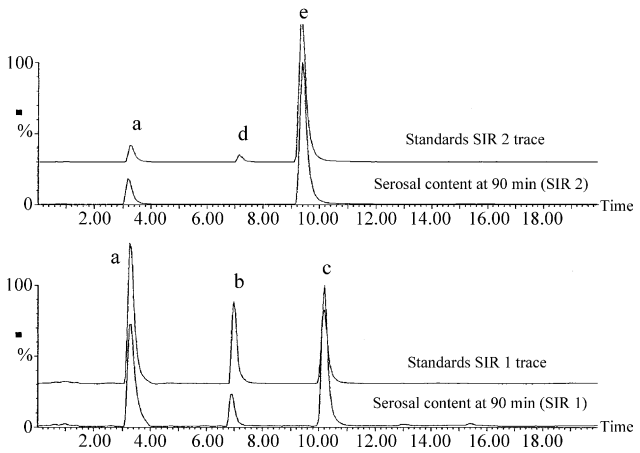

Fig. 3. Selected ion recording (SIR 1 and 2) chromatograms of the serosal content (inside) of an everted gut sac after incubation for 90 min in TC 199 with dextromethorphan (100 μM) on the mucosal side (outside) compared with chromatograms of standard compounds: (a) IS (codeine); (b) dextrorphan; (c) 3-methoxymorphinan; (d) 3-hydroxymorphinan; and (e) dextromethorphan.

of the sac in a 100 μM dextromethorphan solution in TC 199 medium for 90 minutes and extracted as described in Section 2. The major metabolites 3-methoxymorphinan and dextrorphan were detected in the serosal medium as can be seen by comparing the retention times with those of standards of the metabolites. 3-hydroxymorphinan was not detected in the samples but this minor metabolite was also not found by Yu and Haining [13] using human cDNA-expressed CYP4502D6 in vitro. As can be seen in Fig. 4, 3-methoxymorphinan and traces of dextrorphan was also found in the mucosal media suggesting that metabolites exited on each side of the intestinal epithelium. Dextromethorphan, 3-methoxymorphinan and dextrorphan were quantified in the gut sac medium from the serosal side (Fig. 5). In addition

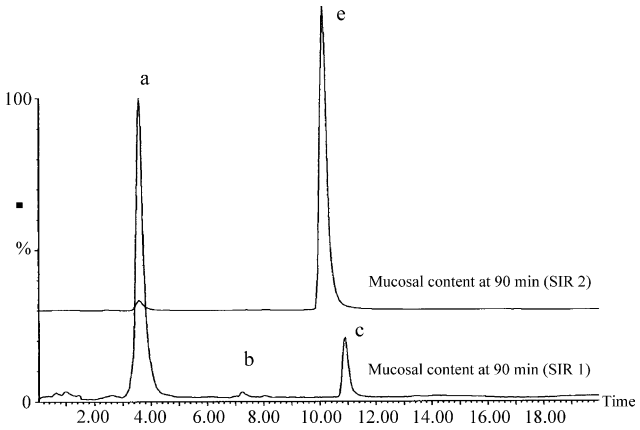

Fig. 4. Representative chromatograms of the mucosal medium (outside) an everted gut sac after incubation for 90 min in TC 199 with dextromethorphan (100 μM) on the mucosal side (outside) showing the appearence on the SIR1 trace of (b) dextrorphan and (c) 3-methoxymorphinan; peaks at retention time of 3.9 and 10.2 min were, respectively, IS and (e) dextromethorphan.

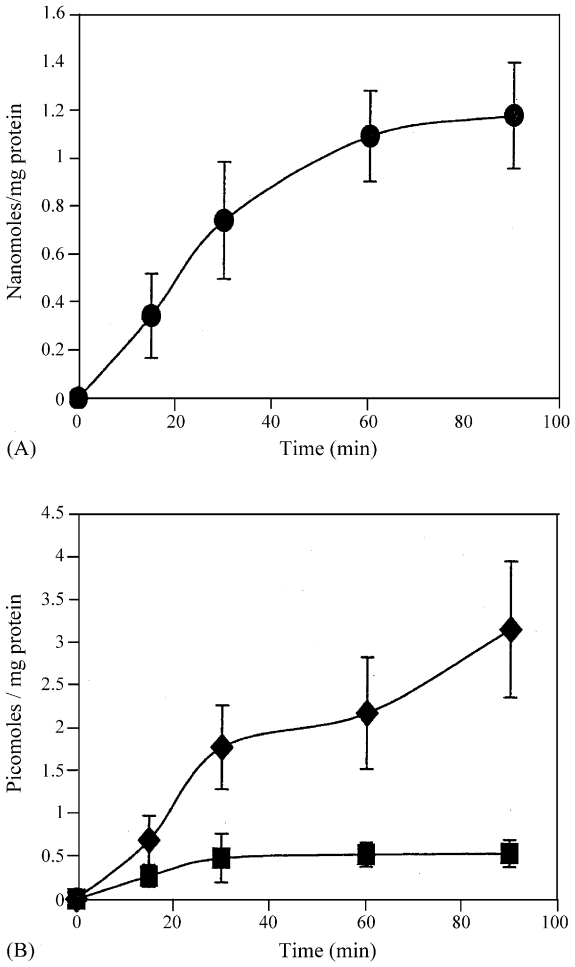

Fig. 5. (A) Accumulation of dextromethorphan (●) inside (serosal content) everted gut sacs incubated at 37 °C in TC 199 medium containing 100 μM dextromethorphan. (B) Accumulation of dextrorphan (■) and 3-methoxymorphinan (♦) inside (serosal content) everted gut sacs incubated at 37 °C in TC 199 medium containing 100 μM dextromethorphan. Each point is the mean ± SD of six sacs, three sacs from two independant experiments (two rats).

3-methoxymorphinan was detected and quantified in the medium of the mucosal side. This is shown in Fig. 6.

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 showed with testosterone [24] and methadone [27], two substrates of the principle cytochrome P450 drug metabolising enzyme isoform 3A, that the rat gut sac model was a valuable model to study intestinal metabolism of such substrates and here we extend the studies to dextromethorphan, a CYP2D1/6 substrate.

The small quantities of material available from in vitro methods meant that the analytical system needed to be very sensitive, accurate, and discriminatory as well as capable

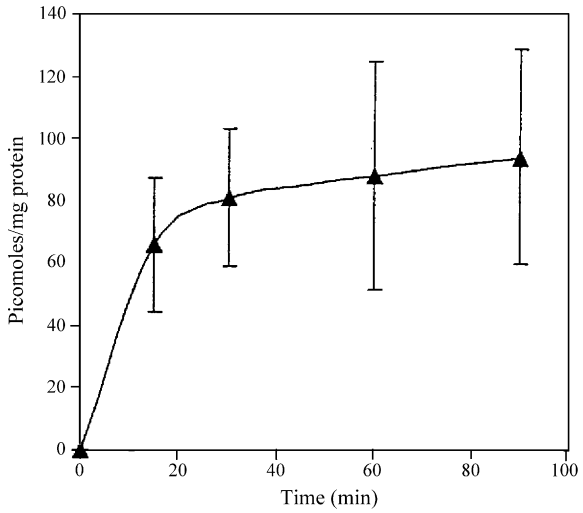

Fig. 6. Appearence of 3-methoxymorphinan in the mucosal medium of everted gut sacs incubated at 37 °C in TC 199 medium containing 100 μM dextromethorphan. Data is expressed as the picomoles transported per mg of tissue protein. Each point is the mean ± SD of six sacs, three sacs from two independent experiments (two rats).

of analysing the metabolites in the presence of tissue culture medium, which contains a wide variety of chemical components. The LC–MS method described fulfills the requisite criteria with a good recovery of all compounds after sample preparation. The procedures generally reported for dextromethorphan extraction from biological fluids (plasma, urine) consist of an initial liquid–liquid extraction followed by a back extraction of the organic layer by an aqueous buffer that is directly injected for analysis [34,36]. This strategy is not suitable for our samples because we have to concentrate the extracted residue for the quantification of metabolites. The extraction procedure was therefore optimised for the simultaneous extraction of dextromethorphan, the internal standard, and the three metabolites in TC199. While their physico-chemical properties might favour the extraction of dextromethorphan and 3-methoxymorphinan in basic conditions, acidification of the samples to pH 6 increased the extraction yield of the internal standard without significantly decreasing the extraction efficiency for dextromethorphan, dextrorphan and 3-methoxymorphinan. Dextromethorphan and 3-methoxymorphinan were adequately extracted by dichloromethane after acidification of the samples (addition of 5 μL of 10% acetic acid, pH 6), but dextrorphan was poorly extracted (around 50%) and hydroxymorphinan was not recovered at all by this procedure. On the contrary, hydroxymorphinan was extracted in 1-butanol whereas the other compounds were not satisfactorily extracted by this solvent. The procedure finally devised consisted of a first extraction with dichloromethane/hexane (1/1, v/v) followed by a second extraction with a mixture of dichloromethane/1-butanol (2/1, v/v) in acidic conditions. This successfully extracted all compounds with extraction recoveries greater than 85% with the exception of 3-hydroxymorphinan at very low concentration (75%). The best analytical method in terms of sensitivity and time economy is LC–MS/MS spectrometry and some methods using this technology have been reported to measure dextromethorphan and dextrorphan in plasma with a good sensitivity. For example, Eichhold et al. [30] proposed an LC–MS/MS method to quantify dextromethorphan and dextrorphan in plasma with a LOQ of 5 pg/mL (0.1 pg on the column) but the two analytes were not quantified simultaneously because the method used two different mobile phase conditions in order to eluate each compound in 1.1 min. They used a liquid–liquid procedure for sample preparation as was generally the case for biological matrices but the sensitivity of the method was decreased when the extraction procedure was replaced by a “dilute-and-shoot” procedure, which consisted of diluting samples without extraction and gave a LOQ around 2 ng/mL (0.3 pg on the column) for dextromethorphan [31]. An LC–MS/MS method could also dramatically increase the speed of analysis, however, a good chromatographic separation from the biological matrix components is essential for obtaining accurate and reproducible data [32]. Whereas the speed and sensitivity of the LC–MS method we present herein were inferior to the two methods cited above, our method was able to simultaneously quantify dextromethorphan and its three major metabolites and was a thousand times more sensitive than the LC–fluorescence method of Hendrickson et al. [34] and considerably more sensitive than the LC–MS/MS quantification described by Vengurlekar et al. [39] who reported a LOQ of 1 ng/mL (0.01 ng injected) for dextromethorphan, and 3-methoxymorphinan, 60 ng/mL (0.6 ng injected) for dextrorphan and 100 ng/mL (1 ng injected) for 3-hydroxymorphinan. Furthermore, the sensitivity of our method was in the same range as the LC–ESI–Ion trap MS method reported for the quantification of dextromethorphan (LOD: 0.25 nM, 12.9 pg injected) and dextrorphan (0.25 nM, 1.35 pg injected) by Zhang et al. [33].

Our results with dextromethorphan confirm the data obtained from the metabolism study of testosterone with the rat intestine everted gut sac technique and validated this technique for studying drug metabolism in vitro. In this method, the tissue is incubated in oxygenated tissue culture medium, which ensures maximum viability and metabolic activity, with the test drug on the mucosal side. As the drug is absorbed across the intestinal mucosa it will appear in the serosal medium inside the sac along with any metabolites formed by cytochrome P450 activity during the passage of the molecule. Thus the method gives a direct indication of the extent of first pass metabolism in the intestinal mucosa. In addition, metabolites can be measured in the mucosal medium, which may indicate activity of efflux transporters in the apical cell membranes. Several such transporters are documented with the predominant one being P-glycoprotein (P-gp) which has a very broad substrate specificity.

As discussed earlier in the introduction, there are considerable differences in the literature concerning which isoforms of the P450 enzymes are present in the rat intestine. In our previous studies with testosterone and methadone [24,27], we detected the formation of 6β-OH-testosterone

and 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine respectively, two P4503A/1A products, showing that these isoforms are active in the mucosal cells, and in the present study we detected the formation of 3-methoxymorphinan, another P4503A product, thus confirming our earlier observation. In addition we also detected dextrorphan, a 2D6/1 product, indicating the presence of enzymes of the 2D group and thus our results showed that both *O*- and *N*-demethylation activities are present in the rat intestine. This has recently been corroborated by studies in our laboratory (submitted for publication) using microsomes prepared from rat intestine, where as well as 3A-type activity, 2B, 2C and 2D activities were detected using specific substrates.

We have also calculated the apparent permeability (P_{app}) of dextromethorphan to compare the absorption with other molecules studied with the everted sac system. The value obtained was $5.08 \pm 0.23 \times 10^{-5}$ cm/s, which was similar to the P_{app} we obtained with testosterone ($5.41 \pm 0.51 \times 10^{-5}$) [24], in keeping with the similar lipophilicity of both compounds (calculated log *P*-values of 3.97 and 3.27 for dextromethorphan and testosterone, respectively).

From studies on the effect of grapefruit juice on dextromethorphan pharmacokinetics in man, Di Marco et al. [40] hypothesized that dextromethorphan is a substrate of P-glycoprotein (P-gp) or a related membrane efflux protein of the gastro-intestinal tract. We have not found any reports in the literature to corroborate this hypothesis with respect to the intestine, but a recent study reports that dextromethorphan showed higher accumulation in the brains of mice in which the P-gp gene had been deleted, suggesting that it is a P-gp substrate [41]. Our results, presented in Fig. 6 show that there is an accumulation of the metabolite 3-methoxymorphinan in the mucosal medium, and as the concentration is higher than in the serosal compartment, this suggests that this product may well be expelled from the mucosal cells by an active process such as the transporter P-gp. The graph also shows a plateau which may represent the kinetics of a saturable transport system. Some investigators postulate an interplay between P-gp function and P450 enzyme activity [42] and the everted sac system, coupled with the sensitive analytical methods such as described in this paper, affords an excellent approach to study the interactions between efflux transport and metabolism, particularly using specific inhibitors of P-gp and/or the P450 enzymes. Such studies are now in progress in our laboratory.

In conclusion, we present a sensitive, accurate and versatile LC–MS method for analysing the major metabolites of dextromethorphan in a complex medium. Using this method, we have confirmed that in rat intestine the P4503A family of isoenzymes are active during drug transport and have shown for the first time that the P4502D isoform is also active, but to a lesser extent. Given that the rat is a widely used species for the pre-clinical assessment of drug absorption and metabolism, such studies are very valuable in evaluating the role of mucosal drug metabolism by different enzymes in oral drug bioavailability.

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