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High-performance liquid chromatographic method for the direct determination of 4-methylumbelliferone and its glucuronide and sulfate conjugates

Application to studies in the single-pass *in situ* perfused rat intestine–liver preparation

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ABSTRACT

A direct high-performance liquid chromatographic (HPLC) assay was developed for the separation and determination of 4-methylumbelliferone (4MU) and its glucuronide (MUG) and sulfate (MUS) conjugates in the cell-free perfusate ("plasma") from *in situ* perfused rat intestine–liver preparation. In addition, a procedure was developed to extract and determine 4MU in the whole blood perfusate. Perfusate plasma containing an internal standard (umbelliferone) was precipitated with methanol (1:4, v/v), and injected into a reversed-phase HPLC system with gradient elution. 4MU and the same internal standard were also extracted directly from the whole blood perfusate with ethyl acetate and injected into a reversed-phase HPLC system with isocratic elution. Inter- and intra-day precision studies ($n = 5$ for each) for both the plasma and whole blood procedures demonstrated relative standard deviations of less than 10% at all concentrations studied. The compounds were stable in either the plasma or blood extracts at room temperature for up to 72 h. The procedures were successfully used to analyze perfusate samples obtained from the single-pass *in situ* perfusion of rat intestine–liver system with either trace (0.95 n*M*) or 32.3 μM concentrations of 4MU. The intestine was responsible for the formation of most of the MUG formed by the intestine–liver preparation during steady-state perfusion with either input concentration of 4MU.

INTRODUCTION

4-Methylumbelliferone (7-hydroxy-4-methylcoumarin, hymecromone, 4MU) is a choleretic agent which is used as a model compound for the study of phase II metabolism, specifically glucuronidation and sulfation in first-pass organs [1–5].

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Reports on the metabolism of this compound generally convey that glucuronidation is the predominant pathway in both the intestine [1] and the liver [3]. Recent evidence indicates that sulfation occurs as a high-affinity, low-capacity pathway in the perfused rat liver [5]. In this preparation, low input concentrations of 4MU were highly cleared (extraction ratio >0.95) to form mostly the sulfate (MUS, 82%, 4MU glucuronide, MUG, 18%), with MUG appearing primarily in the bile. MUG was the dominant metabolite found when higher 4MU concentrations were employed [5]. Moreover, MUS and, to a lesser extent, MUG are known to undergo futile cycling with 4MU [6,7], a phenomenon which complicates the interpretation of the kinetic data of 4MU conjugation.

The conjugates of 4MU have generally been determined indirectly. MUG and MUS in blood [1], organ perfusion medium [6,8], liver homogenates [6], hepatocytes [8] and microdissected liver sections [6,7] were hydrolyzed to 4MU by β-glucuronidase or arylsulfatase; 4MU was then determined by fluorimetry [1,6–8]. Other investigators have separated 4MU from MUS and MUG in liver or brain homogenates [9], hepatocytes [10] and urine, bile and plasma [11] by either open-column chromatography with Dowex AG-50W resins [9,10] or thin-layer chromatography [11]. 4MU was determined by fluorimetry after the individual conjugates had been eluted from the columns or plates, and the fractions containing MUG and MUS were hydrolyzed by β-glucuronidase or arylsulfatase [10,11] or by heating at 80°C for 30 min [9]. A high-performance liquid chromatographic (HPLC) assay for the determination of 4MU and its conjugates in human plasma has also been reported, in which an ion-pair reversed-phase system with UV detection at 254 and 280 nm was used. However, MUS was externally added to each plasma sample as a means to standardize the recovery of 4MU and MUG from the sample [12].

In order to address adequately the relative importance of the intestine and liver in the metabolism of 4MU, a more sensitive HPLC assay with internal standardization was developed for the direct determination of 4MU, MUS and MUG. The *in situ* perfused rat intestine–liver preparation was used to investigate the individual roles of the organs in the conjugation of 4MU. A red cell–albumin-based perfusate was used for perfusion of [^{3}H]4MU (0.94 n*M*) and unlabeled 4MU (32 μM). 4MU and its conjugates in influent and effluent blood perfusate (reservoir, portal venous and hepatic venous) and derived plasma perfusate and bile were quantified by the newly developed methods. This paper reports the validation of the HPLC methods for the determination of unlabeled drug and metabolites, and preliminary results of the perfused rat intestine–liver preparation. As radiolabeled 4MU was employed in the perfusion studies, the metabolites were separated by HPLC and fractions were collected, followed by liquid scintillation counting.

EXPERIMENTAL

Chemicals

Bovine serum albumin (BSA, 25% in Tyrode's buffer), 4MU, MUG and MUS were obtained from Sigma (St. Louis, MO, U.S.A.), as was umbelliferone (UMB), the internal standard. [^{3}H]4MU (non-specifically labeled, specific activity 31.8 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). [^{3}H]4MU was extracted into hexane, then purified by thin-layer chromatography. The radiochemical purity was >97%, as verified by HPLC. HPLC-grade methanol was purchased from Caledon Labs. (Georgetown, Ont., Canada) and HPLC-grade ethyl acetate from Fisher Scientific (Springfield, NJ, U.S.A.). Dextran T-40 was obtained from Pharmacia (Piscataway, NJ, U.S.A.). Dextrose (50%) was obtained from Abbott Labs. (Montreal, Que., Canada). The calcium chloride (analytical-reagent grade) for the Krebs–Henseleit buffer was purchased from BDH (Toronto, Ont., Canada). All other buffer reagents were purchased from Fisher Scientific and were of analytical-reagent grade. Outdated whole human blood was obtained from the Red Cross (Toronto, Ont., Canada).

Preparation of calibration sets and samples for validation of the plasma assay of 4MU, MUS and MUG

Perfusion medium was made up of 20% washed human red blood cells, 1% BSA, 3% dextran T-40, 0.3% dextrose in Krebs–Henseleit hydrogencarbonate solution (pH 7.4) [13]. For the plasma assay, separate calibration sets for 4MU, MUG and MUS were prepared in cell-free medium (referred to subsequently as plasma). A separate 50-μl aliquot of each standard solution (1–200 μg/ml of 4MU, MUS or MUG in methanol) was added to 100 μl of plasma in 1.5-ml microcentrifuge tubes (Fisher, Mississauga, Ont., Canada). After the addition of 25 μl of internal standard (umbelliferone, 100 μg/ml in methanol), the tubes were vortex mixed. The blank consisted of 100 μl of plasma. An additional 325 μl of methanol were added to each standard, while 400 μl of methanol were added to the blank for the precipitation of albumin. All the tubes were vortex mixed, stored at 0°C for 30 min, then centrifuged in a microcentrifuge (Microfuge; Brinkman, Mississauga, Ont., Canada) at 6000 *g* for 2 min. From the supernatant of each sample, 200 μl were removed and evaporated to dryness under nitrogen. Each sample was reconstituted in 200 μl of 25% methanol in water, then recentrifuged for final removal of protein. The supernatant was transferred into a 250-μl insert for placement in an autosampler vial. Injections of 50 μl were made. To determine the intra-day variability, five calibration sets for each compound were prepared and injected on the same day. The inter-day variability was determined by preparing a calibration set for each compound on five different days, with injection of each set on the day it was prepared.

To determine whether 4MU and the metabolites were stable at room temperature after precipitation, one set of the inter-day calibration solutions was in-

jected, stored at room temperature for 48 h, then re-injected. This calibration set was then stored at room temperature for an additional 24 h, then re-injected.

Preparation of calibration sets and samples for validation of the blood assay of 4MU

Calibration sets were also prepared for validation of the determination of 4MU in perfusion medium which included red blood cells (referred to subsequently as blood). A 50-μl volume of 4MU (2–4000 μg/ml in methanol) was added to 1.95 ml of blank blood in 15-ml polypropylene tubes (Sarstadt, Montreal, Que., Canada) to give final concentrations of 0.05–100 μg/ml. The internal standard (30 μl of a 1 mg/ml solution of umbelliferone in methanol) was added, mixed, then 10 ml ethyl acetate were added and the tubes were vortex mixed. The mixture was centrifuged at 2000 *g* for 10 min. The ethyl acetate layer was removed, evaporated to dryness under nitrogen and reconstituted in 200 μl of methanol. The methanol solution was transferred into a 250-μl microcentrifuge tube, centrifuged at 6000 *g* for 2 min, then transferred into a 250-μl insert for placement in an autosampler vial. Injections of 5–10 μl were made. To determine the intra-day variability, five calibration sets were prepared and injected on the same day. The inter-day variability was determined by preparing a calibration set on five different days, with injection of each set on the day it was prepared.

The stability of 4MU after extraction was determined by storing one set of the inter-day calibration solutions at room temperature for 48, 72 and 96 h, then re-injecting them at the specified times.

HPLC conditions

The Waters Assoc. (Mississauga, Ont., Canada) HPLC system included M-6000A and M-45 solvent delivery systems, a Model 440 UV detector, a WISP 710B autoinjector, an M-730 data module and a Model 720 system controller. Mobile phase was pumped at a flow-rate of 1.0 ml/min through a Waters Assoc. μBondapak C_{18} (10 μm) column (30 cm × 3.9 mm I.D.) which was preceded by a guard column containing C_{18}/Corasil Bondapak (particle size 37–50 μm). Detection was effected at 313 nm. For the plasma assay gradient elution was required. Mobile phase A consisted of 25% methanol in 0.05 *M* KH_2PO_4 and mobile phase B was methanol. The initial conditions of 25% methanol in 0.05 *M* KH_2PO_4 were held for 8 min, then a gradient to 40% methanol was developed over 2 min following a linear gradient, and held at these final conditions until 25 min. The system returned to the initial conditions over 2 min, then re-equilibrated until 40 min, at which time the next injection could be made.

Isocratic conditions were used for the assay of 4MU in blood. The same HPLC system and column as described above were used, but the mobile phase was 40% methanol in water with a flow-rate of 0.8 ml/min.

Rat intestine–liver perfusion

The surgical preparation of the *in situ* vascularly perfused rat intestine and liver was carried out as reported earlier [14]. The intestine–liver preparation was perfused in a single-pass manner for 80 min with either trace concentrations (0.95 n*M* containing 20 μCi of [^{3}H]4MU in 600 ml) or 32.3 μM of 4MU (containing 20 μCi of [^{3}H]4MU in 600 ml). Perfusate samples were taken from the hepatic venous outflow at 10, 20, 30, 40, 50, 60, 70 and 80 min and from the pyloric vein at 5, 15, 25, 35, 45, 55, 65 and 75 min. Perfusate samples were handled as described above. Bile was also collected at 10, 20, 30, 40, 50, 60, 70 and 80 min, diluted 1:10 or 1:50 in water, and injected directly into the HPLC system with gradient elution. Fractions were collected from the HPLC eluate of plasma and bile injections at retention times corresponding to those of 4MU, MUG and MUS, as determined in advance by injection of unlabeled standards. After addition of scintillant (Ready-Safe; Beckman, Mississauga, Ont., Canada), the fractions were analyzed using liquid scintillation counting (Beckman Model LS6800). After correcting the fractions for recovery through the precipitation and HPLC steps, the counts in disintegrations per minute (dpm) per milliliter of plasma were converted to nmol/ml with the use of the specific activity of 4MU. Similarly, the counts in dpm per milliliter of bile were converted to nmol/ml. Whole blood perfusate was extracted as described above and 4MU was determined both with the use of a calibration graph and by fraction collection. Data analysis was carried out as described previously [15].

RESULTS AND DISCUSSION

The original procedure for the perfusate plasma involved precipitation of the albumin with 1 *M* perchloric acid, prior to direct injection into the HPLC system. However, subsequent validation studies indicated that MUS was unstable in this acidic medium. Precipitation of the plasma with an organic solvent such as methanol (1:4, v/v) was pursued as an alternative. Although perfusate plasma is relatively "clean", similar methods can be used for blood or plasma, or any other biological matrix.

The methanol precipitation and subsequent HPLC analysis displayed excellent reproducibility in both the inter-day (Table I) and intra-day precision (Table II) studies. Over the range 0.05–10.0 μg of 4MU, MUS or MUG added, the relative standard deviations (R.S.D.) of the peak-area ratios were less than 10% in all instances. In addition, the R.S.D.s appeared to be independent of concentration. The individual calibration graphs were linear over the concentration range of interest.

The three compounds were stable in the supernatant from the methanol precipitation at room temperature for up to 72 h (Table III). The R.S.D.s for the same calibration set injected on days 1, 3 and 4 were 10% or less. Fig. 1 depicts the chromatogram of (A) a blank perfusate plasma sample and (B) a perfusate

TABLE I

INTER-DAY PRECISION (n = 5) FOR 4MU, MUS AND MUG DETERMINATION IN 100 μl OF PERFUSATE PLASMA

Amount added (μg)	4MU		MUS		MUG	
	Peak-area ratio (mean ± S.D.)	R.S.D. (%)	Peak-area ratio (mean ± S.D.)	R.S.D. (%)	Peak-area ratio (mean ± S.D.)	R.S.D. (%)
0.05	0.028 ± 0.0026	9.34	0.011 ± 0.0003	2.80	0.009 ± 0.0002	2.67
0.1	0.047 ± 0.0030	6.45	0.018 ± 0.0005	2.98	0.019 ± 0.0013	6.88
0.5	0.200 ± 0.0062	3.11	0.095 ± 0.0061	6.39	0.104 ± 0.0061	5.87
1.0	0.425 ± 0.0081	1.92	0.184 ± 0.0056	3.05	0.198 ± 0.0170	8.59
5.0	2.475 ± 0.0223	0.90	0.985 ± 0.0361	3.66	0.931 ± 0.0219	2.36
10.0	4.707 ± 0.1211	2.57	1.864 ± 0.0540	2.90	1.818 ± 0.1109	6.10

plasma sample containing MUG (6.8 min), MUS (13.9 min), umbelliferone (18.0 min) and 4MU (22.6 min).

In order to determine 4MU in the whole blood perfusate, the described procedure was developed to extract 4MU from the blood. To validate this procedure, precision and stability studies similar to those for the perfusate plasma were carried out. The inter-day precision over a 2000-fold range and the intra-day precision over a 400-fold range were *ca.* 8% or less (Table IV). The calibration graphs were linear over the range 0.05–100 μg/ml of blood. Fig. 2A depicts the chromatogram of a blank whole blood perfusate sample containing the internal standard, umbelliferone, and Fig. 2B the chromatogram from extracted perfusate spiked with 5 μg/ml of 4MU and the internal standard.

TABLE II

INTRA-DAY PRECISION (n = 5) FOR 4MU, MUS AND MUG DETERMINATION IN 100 μl OF PERFUSATE PLASMA

Amount added (μg)	4MU		MUS		MUG	
	Peak-area ratio (mean ± S.D.)	R.S.D. (%)	Peak-area ratio (mean ± S.D.)	R.S.D. (%)	Peak-area ratio (mean ± S.D.)	R.S.D. (%)
0.05	0.026 ± 0.0018	6.81	0.011 ± 0.0004	3.74	0.010 ± 0.0010	9.45
0.1	0.050 ± 0.0013	2.63	0.020 ± 0.0017	8.78	0.018 ± 0.0011	6.22
0.5	0.197 ± 0.0132	6.67	0.090 ± 0.0063	6.93	0.096 ± 0.0054	5.66
1.0	0.656 ± 0.0254	3.87	0.177 ± 0.0106	5.98	0.184 ± 0.0051	2.76
5.0	2.353 ± 0.1499	6.37	0.964 ± 0.0516	5.36	0.889 ± 0.0511	5.75
10.0	4.502 ± 0.2843	6.31	1.811 ± 0.0554	3.06	1.718 ± 0.1594	9.28

TABLE III

STABILITY OF 4MU, MUS AND MUG AT ROOM TEMPERATURE FOR 72 h AFTER PRECIPITATION OF PLASMA WITH METHANOL (1:4, v/v) (n = 3)

Amount added (μg)	4MU		MUS		MUG	
	Peak-area ratio (mean ± S.D.)	R.S.D. (%)	Peak-area ratio (mean ± S.D.)	R.S.D. (%)	Peak-area ratio (mean ± S.D.)	R.S.D. (%)
0.05	0.028 ± 0.0019	6.78	0.012 ± 0.0008	6.46	0.011 ± 0.0011	10.17
0.1	0.048 ± 0.0032	6.70	0.025 ± 0.0010	4.23	0.023 ± 0.0005	2.30
0.5	0.199 ± 0.0021	1.04	0.096 ± 0.0058	6.04	0.110 ± 0.0037	3.36
1.0	0.481 ± 0.0205	4.25	0.174 ± 0.0026	1.49	0.232 ± 0.0089	3.85
5.0	2.486 ± 0.0188	0.76	0.979 ± 0.0017	0.18	0.928 ± 0.0044	0.47
10.0	4.987 ± 0.0426	0.86	1.919 ± 0.0202	1.05	1.787 ± 0.0170	0.95

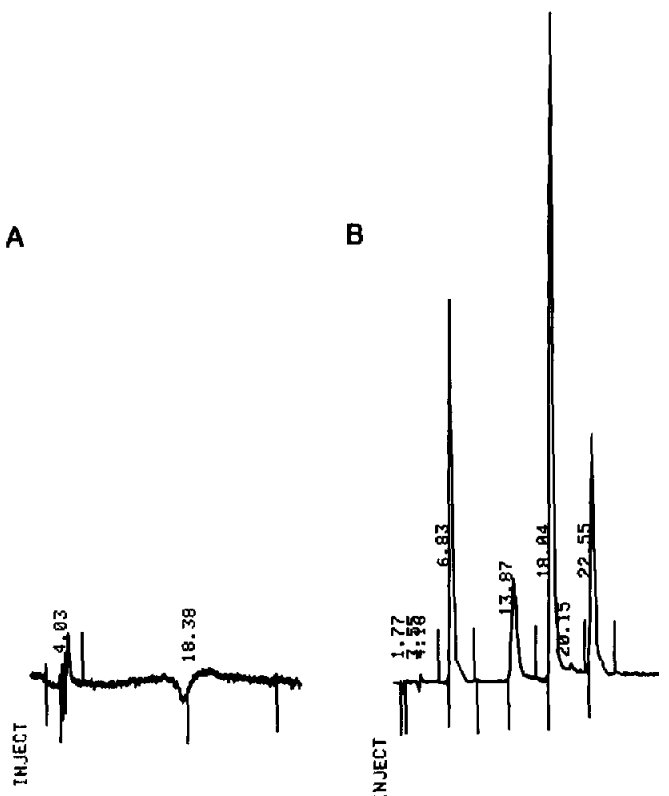

Fig. 1. (A) HPLC of a blank perfusate plasma sample after precipitation with methanol (1:4, v/v). (B) HPLC of a perfusate sample containing MUG (6.83 min), MUS (13.87 min), umbelliferone (I.S., 18.04 min) and 4MU (22.55 min), after precipitation with methanol (1:4, v/v).

TABLE IV

INTER- AND INTRA-DAY PRECISION (n = 5) FOR 4MU IN BLOOD-BASED PERFUSION MEDIUM

Final concentration (μg/ml)	Inter-day		Intra-day	
	Peak-area ratio (mean ± S.D.)	R.S.D. (%)	Peak-area ratio (mean ± S.D.)	R.S.D. (%)
0.05	0.0076 ± 0.0004	5.92	N.D.[a]	
0.1	0.0095 ± 0.0002	2.34	N.D.	
0.25	0.021 ± 0.0017	8.19	0.023 ± 0.0013	5.48
0.5	0.036 ± 0.0011	3.18	0.038 ± 0.0019	4.95
5.0	0.292 ± 0.0082	2.79	0.286 ± 0.0058	2.01
15.0	0.896 ± 0.038	4.28	0.830 ± 0.025	3.06
25.0	1.401 ± 0.066	4.72	1.336 ± 0.041	3.09
50.0	2.825 ± 0.207	7.32	2.467 ± 0.100	4.04
100.0	5.009 ± 0.210	4.19	4.779 ± 0.093	1.95

[a] N.D. = not determined.

The stability of 4MU in the reconstituted extract at room temperature for up to 96 h is indicated in Table V. The variability of the peak-area ratios was unacceptably high at 0.25 μg/ml when the extract was stored at room temperature for 96 h. However, if the extracts were kept at room temperature for only 72 h, the R.S.D.s were *ca.* 5% or less. It is clear that the variability is greater at lower than at higher concentrations, but the stability is adequate within a 72-h storage period at room temperature. It is expected that on refrigeration of the samples, an additional improvement in stability would be observed.

The metabolism of [^{3}H]4MU (0.95 nM) and [^{3}H]4MU with bulk 4MU (32.3 μM) in the single-pass *in situ* perfused rat intestine–liver preparation was then studied with the validated HPLC procedures. Constancy of the efflux rate into the portal and hepatic veins and bile was observed at *ca.* 35 min (Figs. 3 and 4). The steady-state intestinal extraction ratio (E_I) was 0.60 at a 4MU input concentration of 0.95 nM, and decreased to 0.36 at 32.3 μM. The corresponding liver extraction ratios (E_H) were high and were fairly constant (0.92 and 0.94) at the two concentrations examined. The estimate of E_I at the higher dose was similar to that (0.4) reported for the rat *in vivo* where 4MU was infused intravenously at a rate of 6.2 μmol/min · kg [1]. The E_H values were also similar to those (0.94–0.96) obtained in the perfused rat liver preparation [5].

At the lower input concentration of 4MU to the intestine–liver preparation, MUG was found to be the dominant metabolite in the portal vein, accounting for 91% of the total metabolites. In the hepatic vein, MUG accounted for about 72% of the total metabolites whereas MUS was about 28%. A substantial amount of 4MU conjugates was excreted into bile (24.7% of the input rate to the liver, found

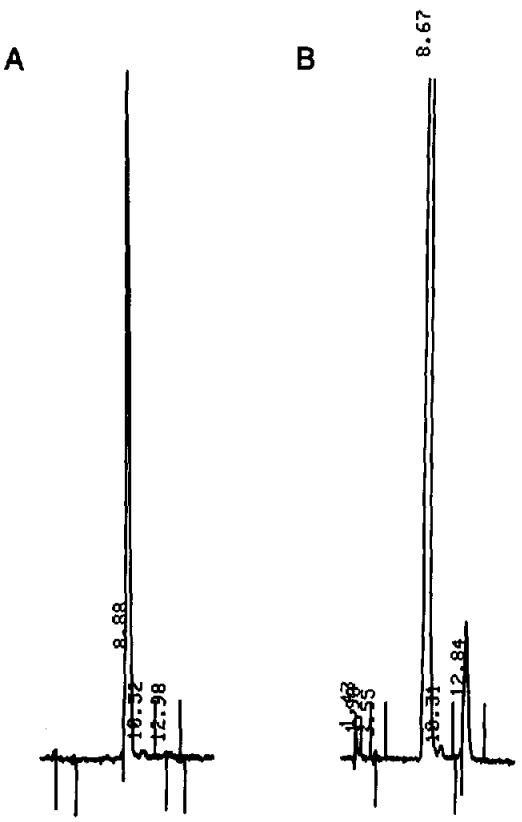

Fig. 2. (A) HPLC of a blank whole blood perfusate sample containing the internal standard, umbelliferone, after extraction with ethyl acetate. (B) HPLC of a whole blood perfusate sample containing the internal standard (I.S., 8.67 min) and 5 μg/ml of 4MU (12.84 min) after extraction with ethyl acetate.

TABLE V

STABILITY OF 4MU AT ROOM TEMPERATURE AFTER EXTRACTION FROM BLOOD-BASED PERFUSION MEDIUM (n = 4)

Concentration (μg/ml)	72 h		96 h	
	Peak-area ratio (mean ± S.D.)	R.S.D. (%)	Peak-area ratio (mean ± S.D.)	R.S.D. (%)
0.25	0.022 ± 0.0012	5.32	0.025 ± 0.0054	21.80
0.5	0.038 ± 0.0011	2.82	0.041 ± 0.0048	11.65
5.0	0.291 ± 0.0026	0.89	0.292 ± 0.0026	0.88
15.0	0.867 ± 0.0013	0.15	0.869 ± 0.0044	0.50
25.0	1.439 ± 0.0032	0.22	1.441 ± 0.0036	0.25
50.0	2.949 ± 0.0044	0.15	2.950 ± 0.0035	0.12
100.0	5.385 ± 0.0473	0.88	5.386 ± 0.0335	0.62

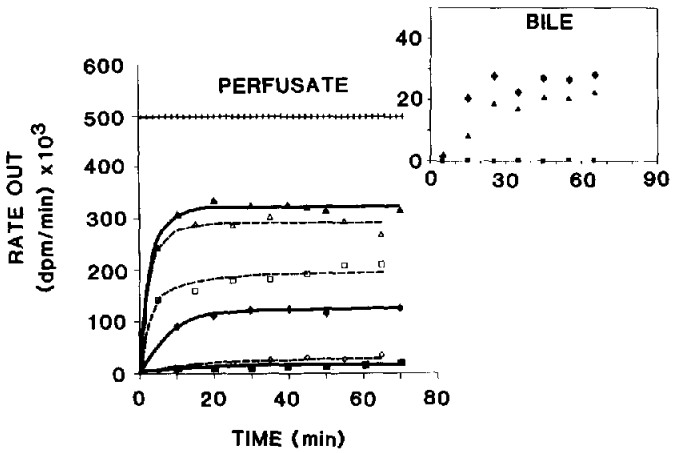

Fig. 3. Rates of efflux (dpm/min) of 4MU, MUS and MUG in portal (open symbols, dashed lines) and hepatic (closed symbols, solid lines) venous perfusate and in bile (inset) at 0.95 n*M* [^{3}H]4MU delivered to the single-pass rat intestine–liver preparation. The rates of efflux into venous perfusate were obtained by multiplying the portal and hepatic venous concentrations by 7.5 and 10 ml/min, respectively. The straight line at the top denotes the rate of input of [^{3}H]4MU into the intestine–liver preparation. (□, ■) 4MU; (◇, ◆) MUS; (△, ▲) MUG.

by concentration of 4MU in portal vein × 7.5 ml/min, the portal venous flow-rate). Of this, <1% appeared as 4MU, with slightly more of MUS (54%) than MUG (Fig. 3). As the luminal contents which contained 4MU, MUS and MUG accounted for <3% of the infused 4MU, the metabolites in the portal vein re-

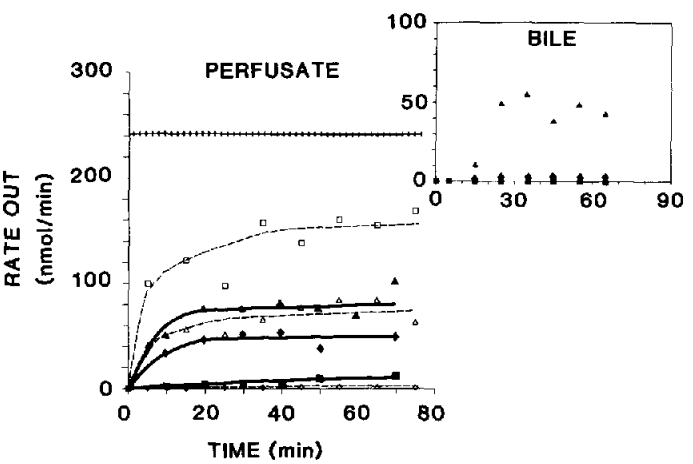

Fig. 4. Rates of efflux (nmol/min) of 4MU, MUS and MUG in portal (open symbols, dotted lines) and hepatic (closed symbols, solid lines) venous perfusate and in bile (inset) at 32.3 n*M* [^{3}H]4MU delivered to the single-pass rat intestine–liver preparation. Symbols as in Figure 3. Note that rates have been converted to nmol/min from dpm/min, as described under Experimental.

flected the intestinally formed metabolites, whereas the sum of the steady-state efflux rates in the hepatic vein and bile reflected the sum of the rates of intestinal and liver metabolism. As intestinally formed conjugates were not further metabolized, the difference between this summed hepatic efflux rate and the portal venous efflux rate may be taken as an estimate of the hepatic conjugation rate of 4MU [15,16]. The hepatically formed metabolite obtained in this fashion was mostly MUS (72.4%). It appears that the preferred reactions for the rat intestine and liver are glucuronidation and sulfation, respectively, with trace input of 4MU of the intestine–liver preparation.

At the higher 4MU concentration (32.3 μM) delivered to the intestine–liver preparation, saturation of the intestinal metabolism was evident with a decreased E_I (0.36); the proportions of MUG and MUS in portal venous plasma were 98.5% and 1.5%, respectively. The proportions of MUG and MUS in the hepatic vein were 65% and 35%, respectively. At the steady state, both MUG and MUS were formed in equal proportions by the liver. About 26% of the input rate of 4MU to the liver was excreted into bile, nearly completely as MUG (94.1%, Fig. 4). The decreased proportions of hepatically formed sulfate and increased proportions of glucuronide at the higher 4MU input concentration (32.3 μM) suggested that saturation of sulfation had occurred in the liver.

The data obtained from this preliminary study of 4MU metabolism by the intestine–liver preparation represents the net effects of conjugation and deconjugation. While there is evidence in the literature that desulfation is a low-affinity pathway that mostly affects futile cycling of MUS at high concentrations [6,7], a similar occurrence for deglucuronidation has not been reported. It remains a challenge to decipher the contributions of desulfation of MUS to futile cycling with 4MU, and to the formation of MUG from MUS via 4MU.

In conclusion, the validation of the analytical methods for 4MU, MUG and MUS indicated excellent linearity, stability and reproducibility after precipitation of perfusate "plasma" with methanol. The extraction of whole blood perfusate and subsequent HPLC analysis for 4MU also indicated excellent linearity, stability and reproducibility. The assay procedure has been utilized to quantify both labeled and unlabeled 4MU, MUS and MUG in perfused liver studies [5]. These methods were also shown to be suitable for the analysis of data generated by the *in situ* perfused intestine–liver preparation.

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