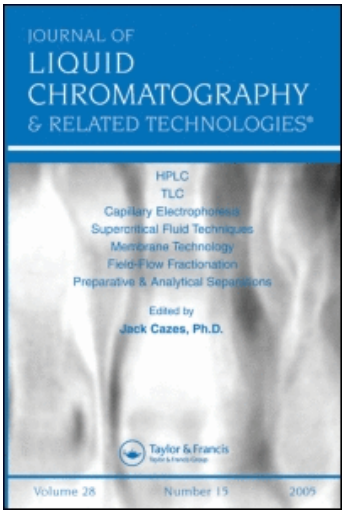

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DETERMINATION OF 4-METHYL UMBELLIFERONE AND METABOLITES IN WILLIAMS E MEDIA AND DOG PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A gradient high performance liquid chromatographic method has been developed for the determination of 4-methylumbelliferone (4MU), 4-methylumbelliferyl sulfate (4MUS), and 4-methylumbelliferyl β-D-glucuronide (4MUG) in dog plasma and William's E media. Samples containing the internal standard, umbelliferone were prepared for analysis by precipitation with acetonitrile prior to injection onto a 150 x 2.1mm C18 Hypersil reversed-phase column. The compounds were eluted in less than 7 minutes with a fast linear gradient from 9.5% acetonitrile to 32% acetonitrile in pH 4.5 acetate buffer with a flow rate of 0.35 mL/min. All compounds were detected at 314 nm. Total sample cycle time was 10 minutes. Intra-run (n=5) and inter-run (n=18) precision was less than 15% relative standard deviation across the entire calibration range. The lower limit of quantitiation with a 50 μL sample size was 2.5 μM.

INTRODUCTION

4-Methylumbelliferone (7-hydroxy-, 4-methyl-coumarin, Hymecromone, 4MU) is a cholerectic agent that is available in Europe [1]. The compound has also been used to treat spasms of the sphincter of Oddi and the biliary ducts [2]. 4MU is almost entirely excreted in the urine or bile of animals as a glucuronide or sulfate conjugate at the 7-hydroxy position [3]. As the compound is highly fluorescent and has a simple pattern of metabolism, it has been used as an attractive model compound to study phase II (conjugative) metabolic pathways for both *in vitro* an *in vivo* drug metabolism experiments.

The glucuronidation of 4MU is catalyzed by UDP-glucuronosyltransferases (UGTs) located in the endoplasmic reticulum of the liver, lung, intestine, and kidney. 4MU is a high activity substrate in microsomal preparations, but is not a specific substrate for any single isozyme. For example, 4MU glucuronidation is catalyzed by at least three isozymes of the human *UGT1* gene family [4]. The *UGT2* gene family specifically catalyzes the glucuronidation of steroids or bile acids, and these isozymes appear to either have low or no activity for 4MU [5,6]. The only isozymes in the *UGT2* family that are capable of catalyzing 4MU glucuronidation are those encoded by *UGT2B8* and *UGT2B11*, however the specific activity appears to be much lower than with *UGT1* isoenzymes [6]. Sulfotransferases are located in the cytosol of various tissues. 4MU is a substrate for the phenol sulfotransferases [3]. 4MU may not be a substrate for isozymes that catalyze sulfation of steroids such as dehydroepiandrostenedione, since other simple phenols such as p-nitrophenol or naphthol are not substrates for steroidal sulfotranserases

[7]. *In vivo*, sulfation appears to be saturable as the excretion of the sulfate conjugate is proportionally lower at higher doses [8].

Quantitative analyses of 4MU, 4MU glucuronide, and 4MU-sulfate have been accomplished by both direct and indirect methods. Indirect methods to quantitatively determine 4MU and its conjugates involve extraction of the unconjugated substrate, 4MU, which can then be measured fluorimetrically (excitation 360-390 nm, emission 450 nm). The conjugates in the aqueous fraction can then be hydrolyzed by treatment with b-glucuronidase/sulfatase, followed by fluorometric determination of the released 4MU. This method has been applied to samples from *in vitro* incubations [9], liver perfusate [10], and whole rat blood [11]. Another approach is to separate 4MU from its sulfate and glucuronide conjugates by ion exchange chromatography on Dowex AG50 resin, followed by hydrolysis of the sulfate conjugate at 80°C for 30 min [12] or by treatment with β-glucuronidase/sulfatase to release 4MU [13]. With the above methods, the sulfate and the glucuronide cannot be independently determined, unless one uses a preparation of β-glucuronidase that is free of sulfatase activity. For example, Anaundi et al. incubated liver perfusate samples with an *E. coli* β-glucuronidase preparation (sulfatase free) to determine the free 4 MU from hydrolyzed 4MUG [10]. To determine the amount of 4MUS, β-glucuronidase activity was inhibited with 10 mM saccharo-1,4-lactone in enzyme preparations from *Helix pomatia* containing both β-glucuronidase and sulfatase, followed by fluorometric determination of the released 4MU [10].

Direct separations of 4MU, 4MUG, and 4MUS have been achieved by thin-layer chromatography and by HPLC. Morita et al. separated the

three compounds on silica gel plates and visualized the spots under UV light at 365 nm [14]. The spots were then scraped, extracted with pH 10.5 Sorensen buffer and 4MUS and 4MUG were hydrolyzed by addition of an equal volume of 2N HCl followed by boiling for 30 min and 2 hr, respectively; 4MU was then measured fluorometrically. Determination of 4MU and it conjugates has also been achieved by HPLC [15-18]. Sandman developed a method for the determination of lysosomal enzyme activity in urine, by determination of the enzymatically produced 4MU from a series of 4MU conjugates including the glucuronide and sulfate [15]. The conjugates were separated from the 4MU on a styrene-divinyl benzene polymer column with a mobile phase consisting of methanol/pH 10.3 glycine buffer. The 4MU was measured on a fluorescence detector. This procedure did not directly measure the conjugates but could be modified for this purpose. Femfert et al. developed a method for the simultaneous determination of 4MU and its conjugates in serum or plasma from humans [16]. This chromatographic method employed tetrabutylammonium bromide as an ion-pairing reagent. Samples (100 µL) were treated with an equal volume of perchloric acid/perchlorate to precipitate proteins and a 10 µL aliquot of the supernatant was injected directly. Compounds were detected at 254 or 280 nm. This method was rapid (less than 9 min), showed good reproducibility, and had a limit of sensitivity of 0.2 µg/mL with a coefficient of variation of 7.5%. More recently, Zimmerman et al. developed a direct method for the determination of 4MU and its conjugates in rat liver perfusate plasma [17]. Sample preparation consisted of addition of umbelliferone as an internal standard to 100 µL plasma samples followed by precipation of perfusate

plasma protein with 4 volumes of methanol. After centrifugation, an aliquot of the supernatant was removed, reconsituted in 200 µL 25% methanol/H20 and injected onto the HPLC column. 4MU and its conjugates were separated on C18 reversed-phase column by gradient elution from 25% to 40% methanol in a phosphate buffer and detected at 313 nm with a total cycle time of 40 min per injection. The sensitivity of this method was 0.5 µg/mL. A separate, more sensitive assay for 4MU in perfusate containing red blood cells that utilized an ethyl acetate extraction procedure was also reported by these authors (limit of quantitation = 0.05 µg/mL). To determine the pharmacokinetics of 4MU and its conjugates in human volunteers, Garrett et al. developed an HPLC method for plasma and urine samples [18]. The compounds were separated on a ODS Hypersil column with an ion-pairing mobile phase similar to that used by Femfert et al. [16]. 4MU was detected by fluorescence (excitation 330 nm, emission 450 nm), 4MUS by UV absorbance at 315 nm, and 4MUG by fluorescence (excitation 330 nm, emission 380 nm). Sample preparation consisted of addition of the internal standard, umbelliferone, followed by an acetonitrile precipitation step. The limit of quantiation for this assay with 20 µL of plasma was 5ng/mL for 4MU, 63 ng/mL for 4MUG, and 409 ng/mL for 4MUS. Sensitivity for 4MU could be increased via an extraction step with larger volumes of sample.

Due to the well documented metabolic profile associated with 4MU, this compound was selected as an *in vivo* and *in vitro* model for phase II drug metabolism in a series of experiments associated with the development of a bioartificial liver at the University of Minnesota [19].

The large number of samples associated with these experiments required the development of an assay with a short analysis cycle time while maintaining accuracy and precision. A sensitive assay was required for the quantitation of 4MU and its conjugates in dog and rabbit plasma and from a modified tisssue culture media that is used to sustain the hepatocytes in the bioartificial liver. Experiments designed to optimize hepatocyte function were conducted with 4MU conjugation as one of the indicators of the performance of the bioartificial liver. As several thousand samples were anticipated, the assay was developed on a small bore column (2.1 mm i.d.) in order to save on solvent costs. A short gradient elution method was selected in order to eliminate late eluting peaks that were encountered with an isocratic, ion-pairing mobile phase.

EXPERIMENTAL

Chemicals

All chemicals were of analytical grade. 4MU, 4MUS, 4MUG, umbelliferone, lidocaine, glacial acetic acid, sodium acetate, and sodium hydroxide were purchased from Sigma (St. Louis, MO). Umbelliferone was recrystallized two times from ethyl acetate to remove a minor impurity. Acetonitrile (Fisher, Fairlawn, N.J.) was HPLC grade. William's E media (Gibco, Grand Island, NY) was supplemented with 200 U/L insulin, 40,000 U/L penicillin G, 400 mg/L streptomycin sulfate, and 0.292 g of L-glutamine per liter. The media also contained 5 or 12 μg/mL of lidocaine and 60 μM 4MU as biotransformation markers.

Instrumentation and Chromatography

Chromatography was performed on a Hewlett-Packard 1090L liquid chromatograph (Palo Alto, CA) outfitted with a column oven, autoinjector, and a diode array detector set at 314 nm with a 10 nm bandwidth for analyte monitoring and 510 nm for reference. Mobile phase was delivered at 0.35 mL/min through a Keystone Scientific (Bellefonte, PA) 5μ C18 Hypersil column, 150 x 2.1 mm i.d., maintained at 40°C in a column oven. The A mobile phase consisted of 0.5% Na acetate buffer, pH 4.5 (prepared by mixing solutions of 0.5% Na acetate and 0.5% acetic acid to pH 4.5) and acetonitrile (19:1 v/v). Mobile phase B consited of 0.5% acetate buffer, pH 4.5 and acetonitrile (1:1 v/v). The initial conditions consisted of 10% B and increased to 60% B in a linear gradient over 6 min which was then maintained at 60% B for an additional 1 min. This was followed by a three min equilibration period at the initial conditions. A personal computer with Chromperfect Direct software (Justice Innovations, Palo Alto, CA) was used to acquire data.

Sample and Standard Curve Preparation

A 50μL aliquot of blank plasma or media or samples of plasma or media containing 4MU and its metabolites was pipetted into a 12 x 75 mm disposable test tube to which 20 μL of a 100 μM solution of umbelliferone (internal standard) in methanol was added. Protein precipitation was accomplished by addition of 2.0 mL of acetonitrile, mixing on a vortex mixer for 10 sec, and centrifugation at 1500 g for 10 min. The acetonitrile was transferred to a clean 10 x 75 mm disposable test tube and evaporated to dryness under N_2 in a 50°C water bath. The residue was

reconstituted in 75 µL of Mobile phase A, transferred to autoinjector vials, and 5 µL was injected onto the column.

RESULTS

4MU, 4MUG, and 4MUS were detected at 314 nm with a diode-array detector. This high wavelength is the UV maximum for the three compounds and is relatively free of interference from potential contaminants in plasma or tissue culture media. Chromatograms of a blank dog plasma extract and a plasma sample from a dog administered 4MU are shown in Figure 1. Figure 2 shows the results obtained from extracts of 50µL samples of a modified William's E media. No interfering peaks were observed under the peaks of interest. The total cycle time for the assay was 10 minutes and the retention times for the peaks of interest were as follows: 4MUG = 2.2 min, 4MUS = 4.0 min, umbelliferone (internal standard, I.S.) = 5.0 min, and 4MU = 6.3 min.

Recovery, Precision, and Accuracy.

Peak heights were used for quantitation. Linear regression of the peak height ratios versus the drug concentration were performed to determine the slope, intercept, and correlation coefficient of the standard curves. Within-run calibration curves consisted of 5 replicates at each level. Between-run calibration standards and quality control standards were assayed in triplicate on 6 different working days.

Recovery was determined by comparing the peak heights of treated plasma and WEM samples with the peak heights of standard injections of the same concentration. Recoveries (mean $\pm$ S.D., n = 5) at 10 nMol/mL

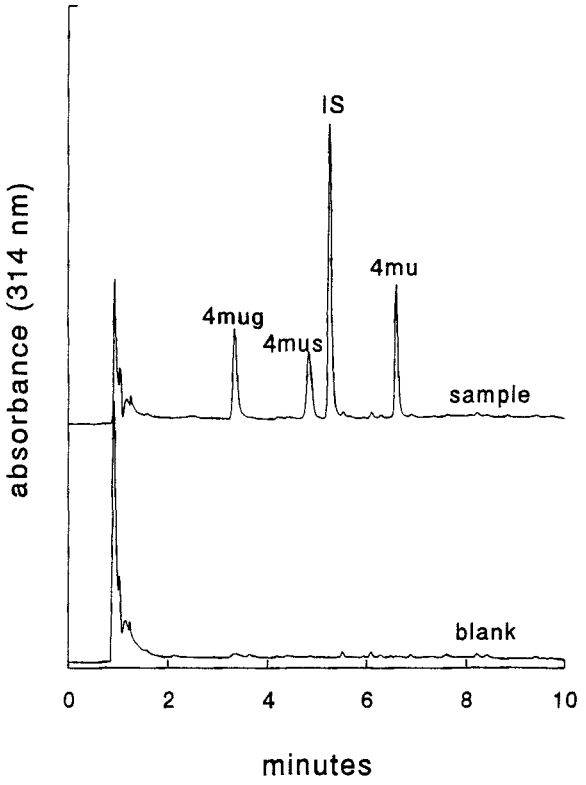

FIGURE 1.

Blank dog plasma and dog plasma sample. Separation occurred on a Keystone ODS (150 x 2.1mm i.d., 5 µm Hypersil) reversed-phase column. The compounds were eluted at a flow rate of 0.35 mL/min with a linear gradient from 9.5% acetonitrile to 32% acetonitrile in pH 4.5 acetate buffer over 6 minutes then maintained at 32% acetonitrile for 1 minute. This was followed by a three min equilibration period at the initial conditions. All compounds were detected at 314 nm.

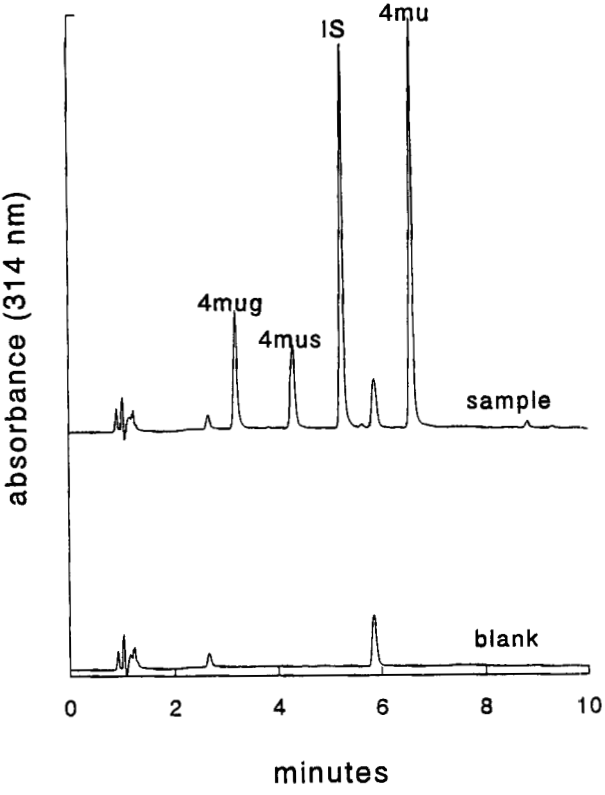

FIGURE 2.

Blank WEM and WEM sample. Conditions as in Figure 1.

in plasma and WEM were respectively 89.4 ± 1.2% and 91.4 ± 1.6% for 4MU, 92.1 ± 7.3% and 91.1 ± 1.1% for 4MUG, 89.4 ± 1.2% and 92.4 ± 2.2% for 4MUS, 88.3 ± 2.3% and 91.6 ± 2.1% for U. Accuracy and precision for the assay in dog plasma and modified William's E media are shown in Tables 1 and 2. Table 1 contains the data for within-run precision (n=5) and Table 2 shows the between-run accuracy and

TABLE 1.

WITHIN RUN PRECISION FOR 4-METHYLUMBELLIFERONE AND METABOLITES IN DOG PLASMA AND WILLIAMS E MEDIA (n=5).

plasma	4mu	rsd	4mug	rsd	4mus	rsd
µMol	avg±sd	(%)	avg±sd	(%)	avg±sd	(%)
2.5	2.50 ± 0.32	12.63	2.45 ± 0.13	5.35	2.53 ± 0.26	10.25
10.0	9.94 ± 0.27	2.73	10.53 ± 0.28	2.68	9.65 ± 0.59	6.16
20.0	20.58 ± 0.61	2.98	21.22 ± 0.68	3.22	19.10 ± 1.14	5.95
50.0	49.96 ± 1.29	2.59	51.10 ± 2.33	4.56	51.57 ± 2.45	4.75
125.0	123.17 ± 4.36	3.54	117.72 ± 3.36	2.86	130.86 ± 6.10	4.66
250.0	248.53 ± 7.71	3.10	235.63 ± 8.48	3.60	247.39 ± 9.56	3.86

media	4mu	rsd	4mug	rsd	4mus	rsd
µMol	avg±sd	(%)	avg±sd	(%)	avg±sd	(%)
2.5	2.46 ± 0.31	12.62	2.49 ± 0.34	13.80	2.46 ± 0.35	14.34
10.0	10.65 ± 0.58	5.49	10.18 ± 0.82	8.06	10.52 ± 0.88	8.38
20.0	20.29 ± 0.78	3.84	19.99 ± 1.68	8.39	20.52 ± 1.75	8.54
50.0	46.59 ± 0.97	2.08	48.73 ± 0.63	1.29	50.22 ± 0.68	1.36
125.0	122.44 ± 5.49	4.48	123.31 ± 4.29	3.48	120.04 ± 4.91	4.09
250.0	255.93 ± 9.44	3.69	256.22 ± 8.10	3.16	243.22 ± 4.97	2.04

TABLE 2.

BETWEEN RUN PRECISION FOR 4-METHYLUMBELLIFERONE AND METABOLITES IN DOG PLASMA AND WILLIAMS E MEDIA (n=18).

plasma	4mu	rsd	4mug	rsd	4mus	rsd
µMol	avg±sd	(%)	avg±sd	(%)	avg±sd	(%)
2.5	2.47 ± 0.21	8.44	2.48 ± 0.25	9.90	2.50 ± 0.18	7.27
10.0	10.19 ± 0.39	3.84	9.76 ± 1.17	11.94	10.43 ± 0.93	8.93
20.0	20.97 ± 0.86	4.11	20.45 ± 1.60	7.83	21.14 ± 1.91	9.05
50.0	51.53 ± 1.86	3.62	53.00 ± 5.91	11.14	52.20 ± 2.31	4.42
125.0	123.20 ± 7.67	6.22	127.34 ± 3.99	10.99	124.10 ± 13.09	10.55
250.0	244.29 ± 10.33	4.23	243.20 ± 25.99	10.69	230.65 ± 5.16	10.91

media	4mu	rsd	4mug	rsd	4mus	rsd
µMol	avg±sd	(%)	avg±sd	(%)	avg±sd	(%)
2.5	2.48 ± 0.18	7.31	2.50 ± 0.38	15.42	2.44 ± 0.23	9.34
10.0	10.21 ± 0.58	5.68	10.05 ± 0.63	6.25	10.64 ± 0.63	5.93
20.0	20.35 ± 0.74	3.65	20.14 ± 1.23	6.11	21.54 ± 1.38	6.39
50.0	49.13 ± 2.14	4.36	49.84 ± 1.47	2.96	51.95 ± 1.45	2.79
125.0	124.24 ± 5.41	4.36	125.68 ± 5.91	4.71	120.41 ± 5.03	4.18
250.0	247.85 ± 10.26	4.14	246.87 ± 15.67	6.35	220.60 ± 8.49	8.38

precision data for the three compounds of interest. The lower limit of quantitation for the three compounds was 2.5 μM. The within-run precision in media at the lower limit of quantitation (2.5 μM) ranged from 10-15% relative standard deviation. At all other concentrations, the precision of the assay was less than 10%.

No degradation of QC samples was noticed over a two week period with samples stored at -70°C. All samples were processed within two weeks of collection and working stocks were made fresh each day.

DISCUSSION

This new assay procedure for 4MU and its metabolites has several advantages over the three previously reported HPLC methods [16-18]. The analysis time is much shorter than the gradient method reported by Zimmerman et al. [17]. For our purposes, detection at 314 nm had adequate sensitivity. The lowest limit of quantitation (2.5 μM = 0.44 μg/mL of 4MU) was comparable to the previously reported methods [15,16] that also used UV detection despite the use of a smaller sample size. Sensitivity for 4MU and 4MUG can be improved with fluorescence detection, as described by Garrett et al. [18], but the emission maxima are different for the two compounds requiring either separate analyses or the availability of a time programmable fluorescence detector. 4MUG and 4MUS display significalntly less native fluorescence than 4MU [15], and consequently, the sensitivity for these compounds with fluorescence detection is not dramatically improved compared to UV detection. Pre

4MU, 4MUG, and 4MUS were detected at 314 nm with a diode-array detector. This high wavelength is the UV maximum for the three

compounds and is relatively free of interference for potential contaminants in plasma or tissue culture media. Chromatograms of a blank dog plasma extract, and extract of dog plasma with the addition of standards, and a plasma sample from a dog administered 4MU are shown in Figure 1. Figure 2 shows the results obtained from extracts of 50µL samples of a modified William's E media. No interfering peaks were observed under the peaks of interest. The total cycle time for the assay was 10 minutes and the retention times for the peaks of interest were as follows: 4MUG = 2.2 min, 4MUS = 4.0 min, umbelliferone (internal standard, I.S.) = 5.0 min, and 4MU = 6.3 min. Accuracy and precision for the assay in dog plasma and modified William's E media are shown in Tables I and II. Table I contains the data for within-day precision (n=5) and Table II shows the between-day accuracy and precision data for the three compounds of interest. The lower limit of quantitation for the three compounds was 2.5 µM. The within-run precision in media at the lower limit of quantitation (2.5 µM) ranged from 10-15% relative standard deviation. At all other concentrations, the precision of the assay was generally less than 10% .

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