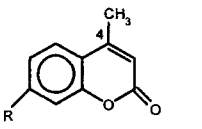
Comparisons of Detections, Stabilities, and Kinetics of Degradation of Hymecromone and Its Glucuronide and Sulfate Metabolites

To the Editor:

In our studies[1] (where drug sources and equipment used are specified in the given reference) on the pharmacokinetics of the anticholeretic and biliary spasmolytic agent hymecromone, information was collected on the previously unknown stabilities and properties of hymecromone (4-MU) and its several metabolites, the glucuronide (4-MUG) and sulfate (4-MUS) conjugates, under various conditions in addition to those published.[1] This material is presented herewith in the text of a communication so as to list such additional factual properties succinctly, without repetition of what had been published[1] (such as sources and materials) and unwarranted expansion of such facts.

4-MU (also known as 4-methylumbelliferone, 4-methyl-7-hydroxycoumarin, or 4-methyl-7-hydroxy-2-oxo-3-chromone; mol. wt. 176.16, Chart 1) had properties similar to other coumarins.[2,3] Its choleretic therapeutic concentrations have been given.[1] Its metabolites have no apparent therapeutic activity.

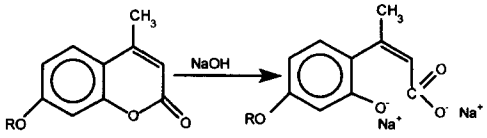


I R = H, Hymecromone, 4-methyl-umbelliferone (4MU)

II R = Glucuronide 4MU glucuronide (4MUG)

III R = Sulfate 4MU sulfate (4MUS)

Chart 1



Coumarin Disodium salt of coumarinic acid

Scheme 1

UV Spectra of 4-MU at Various pH Values—The difference in the acidic maximum of 320 nm (as at pH values of 2.2 and 3.23) and the alkaline maximum of 360 nm (as at pH values of 9.6 and 11.8) with an isosbestic wavelength of 333 nm (invariant absorbances with pH variation) is probably attributable to the dissociation of the 7-phenolic position of 4-MU (Varian, Cary 129 spectrophotometer, Corning pH meter 125).

The 1.0 N NaOH solution of the probable disodium salt of the phenolic coumarinic acid (Scheme 1) had a molar absorptivity of 51 279 at 360 nm. The 1.0 N HCl solution had a molar absorptivity of 25 408 at 318 nm. A 99.2% purity estimate was obtained upon titration.

A 36.7 μM solution of hymecromone at pH 12.2 and 23.9 μM solutions of hymecromone in 0.1 and 1.0 N NaOH (calculated pH values of 12.88 and 13.81, respectively) were monitored spectrophotometrically at 19.5 °C, and their absorbances had isosbestic points (wavelengths of invariant absorbances while 4-MU degrades) of 314–316 and 324 nm, respectively. The maximal 360-nm absorbances of the mono-ionized 4-MU decreased with respective half-lives of 63, 74.5, and 2.54 min (probably due to the formation of the disodium salt of the open-ring hymecromone from the hydrolysis of the coumarinic ring to open the lactone)[2,3] (see Scheme 1). The low 250–326-nm range of absorbances roughly doubled.

There was no observed 4-MU degradation (loss of absorbance) at pH 2.1 over 1000 min, in 1.0 N HCl over 1300 min, at pH 11.7 over 360 min, or in methanol.

The respective maximal molar absorptivities of the acid and neutralized forms of 4-MU were 11 093 and 15 385 with an isosbestic point of 333 nm.

Methanolic solutions of 4-MU (methanol, HPLC grade; Fisher Scientific, Fairlawn, NJ) had two maxima: 218 nm (molar absorptivity of 16 130) and 322 nm (molar absorptivity of 15 270).

Fluorescent Spectra of 4-MU and Its Metabolites—A maximal excitation wavelength of 260 nm (3.76 μM solution) produced fluorescence at pH 7 with maxima of 450 and 363 nm, with the former signal being 4.8 times that of the latter (Spectrofluorimeter Mark A and a Fisher Recordall Series 500, slit width = 2 mm).

The best fluorescent excitation wavelength for un-ionized 4-MU was 335 nm with a maximal emission at 455 nm (from a 51.5 μM solution at pH 4.45). The salt form of 4-MU had a maximum excitation wavelength of 373 nm with a maximal emission of 445 nm. Methanolic solutions had a maximal excitation wavelength of 340 nm, but the maximal emission was at 395 nm and was approximately half that of the aqueous solution (Mark I xenon lamp, Farrand Optical Co.; Fisher Recordall, Series 5000).

Whereas 4-MU with a reversed phase HPLC retention time (t_R) of 8.9 min[1] (330/450-nm detection) and 4-MUG with t_R = 3.9 min (330/380-nm detection) showed good fluorescence,[1] such conditions produced negligible fluorescence for 4-MUS (t_R = 11.9 min). The internal standard 7-hydroxycoumarin had a t_R of 5.8 min. The 4-MUS on HPLC separation was assayed by UV spectrophotometry at 320-nm detection.[1]

Spectrophotometric and Potentiometric pK_a Determinations of Hymecromone—Spectrophotometric pK'_a determination of 4-MU in 1% methanol using HCl, acetate, phosphate, borate, and bicarbonate buffers (prepared from salts and acids obtained from Fisher) permitted the estimation of a pK'_a of 8.0 (±0.21 SD) from the decrease in absorbances at 320 nm and 7.8 (±0.2 SD) from the increase at 360 nm with increasing pH values.

Duplicate potentiometric determinations[4] (radiometer, Copenhagen, with Fisher-certified 1.0 N NaOH and HCl solutions and buffer standards, Fisher Scientific, Fairlawn, NJ) of pK'_a values (±0.1 unit SD) resulted in mean values of 8.5 at 50% methanol–water, 8.3 at 25%, 7.9 at 10%, 7.6 at 5%, and 7.5 at 2.5%.

Spectrophotometric titrations gave pK'_a values of 7.90 and 7.85 (respective molar absorptivities of 11 864 for the undissociated and 15 412 for the dissociated 4-MU) for aqueous 51.5 μM solutions of the monosodium salt of 4-MU at 320 and 360 nm, respectively, and of 7.76 and 7.70 (respective molar absorptivities of 11 093 and 15 385) in 1% methanol–water at the respective wavelengths.

Spectrophotometric titration of aqueous hymecromone, when the disappearance of the 320-nm absorbance was monitored with an increasing pH, gave a pK'_a of 7.90 ± 0.11 SD and, from the appearance of a 360-nm absorbance, pK'_a of 7.85 ± 0.13 SD.

Stability of 4-MU and Its Metabolites—There was no systematic decrease in 4-MU spectral absorbances at constant pH values ranging from 2 to 11 over 20 min. Stable stock solutions of hymecromone were prepared in acetonitrile (with molar absorptivities of 13 535 at the 318-nm maximum and 16 463 at the 215-nm maximum).

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Whereas 4-MU and 4-MUG were stable for more than 1 day in 1.0 N HCl, in plasma, and in urine at 40 °C (and thus at ambient room temperatures where analyses would be conducted) and for at least 4 months in frozen urine and plasma samples, 4-MUS in 1.0 N HCl degraded to 4-MU with a half-life of 135 min at 40 °C.

A more sensitive assay of 4-MUS could be effected after acid degradation of samples and analysis of the resultant increase of 4-MU fluorescence.

The two conjugates were stable in frozen urine and plasma samples for at least 4 months.

The ready transformation of 4-MU in 0.1 and 1.0 N NaOH were mimicked by its conjugates 4-MUS and 4-MUG, which were readily transformed in strong alkali to their corresponding coumarinic acid salts (Scheme 1).

Estimates of Analytical Sensitivity—If analytical sensitivity is defined as twice the standard error (SER) about regression for the linear regression of calibration ($n = 10$, $r > 0.99$), the analytical sensitivity for direct assay of 4-MU in plasma (after acetonitrile deproteinization) was 2.5 ng/mL. When extraction procedures were used,[1] fluorescent analytical sensitivity was heightened to 0.08 ng/mL in plasma and 0.8 ng/mL in urine. The analytical sensitivities for 4-MUG were 50 ng/mL in plasma and 500 ng/mL in urine. The spectrophotometric assay of 4-MUS had a sensitivity of *ca.* 500 ng/mL. Typical calibration regression parameters of ng/mL concentrations, C, vs peak area ratio, PAR, were calculated $C \pm SER = (m \pm s_m)$ $PAR + b \pm s_b$, where s_m is the standard error of the slope and s_b is the standard error of the intercept. The concentration range assayed, the slope ($m + s_m$), and intercept ($b \pm s_b$) values were as follows: for directly assayed 4-MU, 2.6–49.9 ng/mL, 10.00 ± 0.47, −0.60 ± 0.29; for 4-MU after extraction,[1] 0.01–1.00 ng/mL, 0.351 ± 0.021, −0.020 ± 0.008; for 4-MU in urine, 25.0–47.5 ng/mL, 9.78 ± 0.36, 0.06 ± 0.32; for 4-MUG in plasma, 41–273 ng/mL, 264 ± 11.0, −29.4 ± 4.0; for 4-MUG in urine 430 to 7000 ng/mL, 257 ± 8.3, −20.8 ± 4.6. The ranges of concentrations of 4-MU and its metabolites in human plasma can be readily ascertained from the previously published study.[1]

Benzene/Water Partition Coefficients—The benzene/water partition coefficient for 4-MU is *ca.* 1.00 between pH values of 3 and 6, 0.7 at pH 2, 0.9 at pH 7, 0.3 at pH 8.0, and <0.02 at pH 9.0. Extraction of 4-MUG and 4-MUS into benzene from buffered aqueous solutions is negligible at all pH values. These facts indicate that benzene extractions of alkaline solutions[1] at pH 9 could clean up solutions of 4-MU and its metabolites (by removing benzene-soluble containants without disturbing these compounds).

Summary and Significances of These Studies—UV absorbances and maximal UV and fluorescent wavelength data were presented for 4-MU and its metabolites that show that, although 4-MU and 4-MUG can be HPLC analyzed with great sensitivity and specificity using fluorescence detection, this was not feasible with the insignificantly fluorescent 4-MUS, the direct detection of which was limited to spectrophotometry. However, since 4-MUS can be readily degraded to 4-MU in acidic media that do not affect 4-MU or 4-MUG, 4-MUS can be analyzed by the difference in the sensitive fluorimetric assays of 4-MU before and after acid treatment. Spectrophotometrically and potentiometrically determined pK values were determined. Stabilities of 4-MU and its metabolites under various conditions were reported.

References and Notes

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