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DETERMINATION OF 4-METHYLUMBELLIFERONE AFTER SEPARATION FROM ITS CONJUGATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO LYSOSOMAL ENZYME ACTIVITY ASSAYS

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SUMMARY

A high-performance liquid chromatographic method is described for the separation and estimation of 4-methylumbelliferone in the presence of its conjugates. The technique utilizes a simple, isocratic eluent and fluorometric detection. Percentages of 4-methylumbelliferone in the conjugates and fluorescences of the conjugates are reported. 4-Methylumbelliferone, liberated under currently used conditions for enzyme activity in urine and fibroblasts, can be measured by this procedure because these materials contain no substances interfering with the 4-methylumbelliferone peak. Applied to lysosomal enzymatic activity analyses, this procedure eliminates substrate background fluorescence.

Determinations of eight lysosomal enzymes in urine and fibroblasts are presented in bar graph form.

INTRODUCTION

The measurement of lysosomal hydrolases in tissues, cultured fibroblasts, leucocytes, macrophages and body fluids is important for a large number of studies involving both normal and pathological conditions ranging from the role of acrosomal hydrolases in fertilization to diagnosis of genetic deficiency diseases and rejection of a transplanted kidney [1—5]. Following their introduction [6—8], 4-methylumbelliferone (4-MU) conjugates have been used extensively for the estimation of activities of lysosomal glycosidases, lipases, acid phosphatases and arylsulfatases. The determinations are based on the ability of these enzymes to hydrolyze compounds consisting of carbohydrates, lipids, phosphate or sulfate conjugated with 4-MU. Free 4-MU released by enzymatic action is readily estimated by fluorometry, a technique which

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possesses the advantage of greatly increased sensitivity over spectrophotometric methods. Aqueous solutions of 4-MU conjugates, however, exhibit considerable fluorescence due in part to the presence of free 4-MU resulting from its incomplete removal after synthesis and/or from spontaneous decomposition of the compounds during storage and in part to native fluorescence of the conjugate itself. The fluorescence of the latter is usually low relative to 4-MU but, as a substrate, it is present in high concentrations and, consequently, makes an appreciable contribution to the blank correction of the assay. Total background fluorescence can exceed fluorescence of 4-MU liberated by the enzyme.

This paper describes a technique for elimination of that part of the background problem due to fluorescence of the conjugate. This is accomplished by separation of 4-MU from the conjugate using reversed-phase high-performance liquid chromatography (HPLC) on a column of alkali-stable adsorbent and a simple alkaline eluent system for maximizing fluorescence of 4-MU.

MATERIALS AND METHODS

4-MU, 4-MU conjugates and ammonia-free glycine were obtained from Sigma (St. Louis, MO, U.S.A.); methanol was supplied by Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Isocratic reversed-phase HPLC was used with a Hamilton 15 × 0.4 cm PRP-1 column (Hamilton, Reno, NV, U.S.A.). The column packing is a 10-μm spherical styrene—divinylbenzene copolymer which is stable at pH values from 1—13 and in salt concentrations up to 0.5 *N*. A Brownlee 3 cm × 4.6 mm RP 8 guard column (Rheodyne, Berkeley, CA, U.S.A.) was placed in line before the PRP-1 column.

The detector was a Gilson Spectra/Glo Model 901 fluorometer with a standard 45-μl flow cell (Beckman Instruments, Mountain View, CA, U.S.A.). The primary filter had a band pass of 330—380 nm with a peak of 360 nm. The band pass of the secondary filter was 430—600 nm peaking at 455 nm. A linear Model 585 recorder was used (Tegal Scientific, Concord, CA, U.S.A.). Relative fluorescence is expressed in arbitrary units derived from peak heights rather than areas.

Eluent A, used for all conjugates except the sulfate, consisted of 0.04 *M* glycine—sodium hydroxide buffer (pH 10.32) in aqueous methanol. This was prepared by adding 300 ml of a stock 0.4 *M* glycine buffer (pH 10.32) to 1700 ml of water, mixing well, then adding 1 l of spectral-grade methanol. The pH was readjusted if necessary. This system did not separate 4-MU from its sulfate. Eluent B, the same concentration of the glycine buffer in a more aqueous solution (20% methanol in water) achieved this separation. Degassed eluent was pumped at the rate of 1 ml/min at room temperature.

The standard curve was prepared with 4-MU dissolved in water. For the determination of free 4-MU, retention times and relative fluorescence, the conjugates were dissolved in water with warming if necessary. Concentrations ranged from 0.5 m*M* for 4-MU-β-galactoside to 5.0 m*M* for 4-MU-sulfate; the remaining concentrations were 1.0 m*M*. Samples were applied to the column by filling a 20-μl stainless-steel injector loop using a plastic syringe.

For estimation of bound 4-MU, the above solutions were diluted 100-fold

with 0.1 *N* hydrochloric acid and aliquots were sealed in glass ampoules which were placed in a heating block at 100°C for the indicated time periods, then cooled and diluted to 0.2 μM for chromatography.

Normal human fibroblasts were grown to confluency in Falcon T-75 flasks in Dulbecco's MEM H21 medium 1 with 10% fetal calf serum. Cell harvest and preparation of the enzyme solution are described in a previous communication [3]. Protein content of the cell supernatant after the final centrifugation was estimated using the method of Lowry et al. [9].

Overnight urine samples were diluted 5-fold or greater in distilled water containing 1 mg/ml bovine serum albumin. Creatinine determinations were performed by the hospital clinical laboratories using a standard automated procedure based on the reaction with picric acid.

Conditions for enzyme analysis are given in Table I.

TABLE I

CONDITIONS OF ENZYME ANALYSIS

Abbreviations used: β-Glu, β-D-glucuronidase (E.C. 3.2.1.31); β-gal, β-D-galactosidase (E.C. 3.2.1.23); α-Gal, α-D-galactosidase (E.C. 3.2.1.22); NAG, β-N-acetyl-D-glucosaminidase (E.C. 3.2.1.30); AS, arylsulfatase (E.C. 3.1.6.1); AP, acid phosphatase (E.C. 3.1.3.2); α-Man, α-D-mannosidase (E.C. 3.2.1.24); α-Fuc, α-L-fucosidase (E.C. 3.2.1.51); 4-MU-β-glu, 4-methylumbelliferyl β-D-glucuronide; 4-MU-β-gal, 4-methylumbelliferyl β-D-galactoside; 4-MU-α-gal, 4-methylumbelliferyl α-D-galactoside; 4-MU-NAG, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide; 4-MU-SO_4, 4-methylumbelliferyl sulfate; 4-MU-PO_4, 4-methylumbelliferyl phosphate; 4-MU-α-man, 4-methylumbelliferyl α-D-mannoside; 4-MU-α-fuc, 4-methylumbelliferyl α-L-fucoside.

Enzyme	Buffer	pH	Substrate
β-Glu	0.1 *M* acetate	4.75	1.0 m*M* 4-MU-β-glu
β-Gal	0.1 *M* acetate	4.50	0.5 m*M* 4-MU-β-gal
α-Gal	0.1 *M* acetate	4.20	1.0 m*M* 4-MU-α-gal
NAG	0.2 *M* citrate phosphate*	4.60	1.0 m*M* 4-MU-NAG
AS	0.1 *M* acetate	5.00	1.0 m*M* 4-MU-SO_4
AP	0.1 *M* citrate	4.00	1.0 m*M* 4-MU-PO_4
α-Man	0.1 *M* citrate	4.75	1.0 m*M* 4-MU-α-man
α-Fuc	0.1 *M* citrate	5.25	1.0 m*M* 4-MU-α-fuc

*See ref. 10.

The total volume of the reaction mixture was 0.5 ml including the 0.05 ml of diluted urine or homogenate used to initiate the reaction. In addition, bovine serum albumin was present at a concentration of 1 mg/ml. Incubations were carried out at 37°C for 15, 30, or 60 min. The reaction was stopped by the addition of 2 ml of 100% methanol which has an advantage over the generally used alkaline glycine-sodium hydroxide buffer in that it precipitates proteins of the reaction mixture thus avoiding the possibility of their adsorption on the chromatography column. Blank values were obtained by adding the 0.05 ml of enzyme source after the methanol. Precipitated protein was removed by centrifuging at 570 *g* for 10 min and 20 μl of the supernatant were chromatographed using eluent A.

RESULTS AND DISCUSSION

Using eluent A, a standard curve was prepared by chromatographing 0.24 to 36 pmol of 4-MU. A linear correlation between relative fluorescence based on peak height and concentration was observed at all levels. Relative fluorescence by least squares linear regression analysis was given by the equation: $3858 \times [4\text{-MU}] + 10$; r^2 was 0.999. The minimum detectable amount having a peak height two times the short-term baseline noise is 6 fmol.

Table II shows retention times of 4-MU and its conjugates using eluent A. Because this solution did not separate 4-MU from its sulfate, retention times of both these compounds are given for eluent B. Table II also lists relative fluorescences of the conjugates. These values, based on peak heights, are given only to indicate that they will add to background fluorescence. They are not equivalents of 4-MU fluorescence because peak height at a given concentration varies inversely with its retention time. The low fluorescent intensity of the superimposed peaks of 4-MU and its sulfate in eluent A should be noted.

Free and bound 4-MU was determined chromatographically before and after acid hydrolysis of freshly prepared solutions of the conjugates. These values are given in Table III as molar percentages based on weight of the conjugate. The quantity of free 4-MU is very low; however, the compound is much more fluorescent than the substrates and these small percentages in the relatively high substrate concentrations employed in an assay make a considerable contribution to blank fluorescence.

TABLE II

RETENTION TIMES AND RELATIVE FLUORESCENCE OF 4-METHYLUMBELLIFERONE AND SOME CONJUGATES

Reversed-phase HPLC with fluorometric detection. All samples were dissolved in distilled water. To hasten solution 4-MU-β-gal and 4-MU-α-fuc were heated to 65°C; 4-MU-NAG to 50°C; and 4-MU-α-gal, 4-MU-α-man and 4-MU-β-glu to approximately 40°C. Unless otherwise noted eluent A was used. The compositions of eluents A and B are given in the Materials and methods section. 4-Methylumbelliferone is abbreviated 4-MU. See Table I for other abbreviations.

Compound	Retention time (min)	Relative fluorescence/nmol
4-MU	6.4	195,800
4-MU-β-glu	3.3	130.5
4-MU-β-gal	9.9	36.5
4-MU-α-gal	10.4	57.9
4-MU-NAG	15.0	20.1
4-MU-SO_4*	6.4	7.3
4-MU-PO_4	1.8	2650
4-MU-α-man	30.5	14.9
4-MU-α-fuc	58.7	10.3
4-MU**	15.8	69,200
4-MU-SO_4**	19.4	0.75

*Peaks of 4-MU and 4-MU-SO_4 are superimposed.
**Eluent B.

TABLE III

FREE AND BOUND 4-MU IN 4-MU CONJUGATES

All compounds were dissolved in distilled water for determination of free 4-MU. To aid solution, conjugates were heated as noted in Table II. Bound 4-MU was determined after hydrolysis as described in the Materials and methods section. Eluent A was used for all compounds except 4-MU-SO_4 which was chromatographed in eluent B. Composition of these eluents is given in the Materials and methods section. Explanations of abbreviations are given in Tables I and II.

Conjugate	Concentration (m*M*	Molar % free 4-MU	Molar % of theoretical 4-MU recovered after hydrolysis for							
			4 h	5 h	6 h	21 h	24 h	28 h	29 h	30 h
4-MU-β-glu	1.0	0.0066	74.4	82.2	87.1		103.7		104.3	105.2
4-MU-β-gal	0.5	0.0009		99.4			103.0	103.8		
4-MU-α-gal	1.0	0.0103★	92.8	94.1			95.6			
4-MU-NAG	1.0	0.0194	92.3	94.5			95.1			
4-MU-SO_4	5.0	0.0036	92.6				99.3		100.3	
4-MU-PO_4	1.0	0.0723	91.6			97.6	93.8			
4-MU-man	1.0	0.0272		101.2			99.7		101.8	
4-MU-β-fuc	1.0	0.0023	96.5				96.1		94.1	

★At room temperature the percentage of free 4-MU in the 4-MU-α-gal solution gradually increased to 0.0199% over a 5-h period.

Following acid hydrolysis of the conjugates, the expected one-to-one molar ratio of 4-MU to conjugate was found for all compounds. With the exception of the β-glucuronide, over 90% of the theoretical amount of bound 4-MU was liberated after a 4—5 h hydrolysis period. By 24 h hydrolysis was essentially complete for all compounds. Results of the hydrolysis experiments show that 4-MU is stable for at least 30 h at 100°C in 0.1 *N* hydrochloric acid.

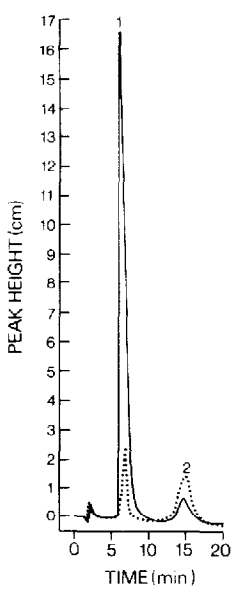

Fig. 1. Chromatogram of urinary NAG assay reaction mixture (——) and assay blank (• • • •). Conditions of analysis and chromatography are described under Materials and methods. Peaks: 1 = 4-MU, 2 = 4-MU-NAG. Enzyme source: 10-fold diluted urine. Detector sensitivity for the assay blank was twice that of the assay reaction mixture.

After a preliminary experiment demonstrated that neither diluted urine nor a fibroblast enzyme preparation in commonly used reaction mixtures would interfere with the chromatography of 4-MU, analyses of eight lysosomal enzymes in urine or fibroblasts were carried out. Fig. 1 shows a typical assay and blank chromatogram from one of these. With the exception of the sulfate, blank corrections involved only fluorescence of free 4-MU in the substrates. From Tables II and III it can be calculated that the 0.0036 molar percent free 4-MU in the sulfate conjugate is responsible for 96% of the fluorescence of their superimposed peaks emerging with eluent A. The remaining 4% was due to fluorescence of 4-MU-sulfate. Therefore, separation of product and substrate with eluent B was not deemed necessary and a blank correction based on their superimposed peaks was used.

Fig. 2 shows, in bar graph form, results of the activity determinations of the enzyme in urine and fibroblasts. The results are in general agreement with those reported previously even though substrate concentrations and pH values of the reaction mixtures differed in most instances [2, 3, 5, 11—14]. The value for urinary acid phosphatase is high reflecting the presence of prostatic enzyme [15, 16]. This activity decreased rapidly over the course of several days when the urine was stored at —20°C.

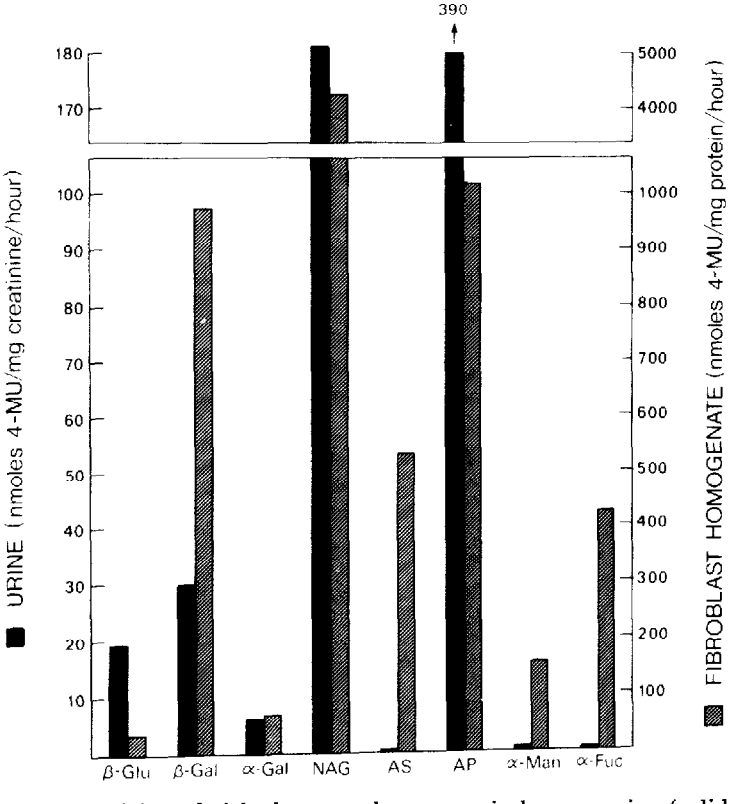

Fig. 2. Activity of eight lysosomal enzymes in human urine (solid bars) and in a fibroblast homogenate (cross-hatched bars). The urinary acid phosphate value given in parentheses above the broken bar includes the prostatic enzyme present in adult male urine. For explanation of abbreviations, see in Tables I and II.

When a long time lapse occurs between the emergence of the 4-MU and a substrate peak, the assay can be shortened by injecting samples during that interval. This technique does not alter the peak height of 4-MU. The timing for these multiple injections can be calculated from retention times of 4-MU and conjugates. As an alternative, late-eluting peaks can be eliminated by substituting a short (3 × 0.46 cm) column for the sample loop. When 4-MU moves to the long column, conjugate retained by the short column is removed with a strong eluent and the column re-equilibrated.

In summary, a method is presented for the estimation of 4-MU in enzyme reaction mixtures commonly used for the determination of lysosomal enzyme activities. The technique is accurate, relatively rapid and eliminates part of the background fluorescence encountered in analyses where 4-MU conjugates are used as substrates. Because only 20 μl or less of reaction mixture need be chromatographed, the method can easily be adapted for use as a microassay.

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