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# High-performance liquid chromatography of the fluorescent dyes Fura-2 and Mag-Fura Stability in organic solvents

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**Abstract**

The fluorescent dye indicators Fura-2/AM and Mag-fura are used to estimate changes in intracellular concentrations of calcium and magnesium, respectively. HPLC, coupled to fluorescence and ultraviolet detectors, indicated that these dyes are unstable when dissolved in methanol and are especially unstable in glass containers compared to plastic. Both dyes were very stable in acetonitrile and dimethyl sulfoxide. The glass vials used in these studies appear to contain an unidentified substance which causes changes in the spectral intensities of these fluorescent dyes in the presence of methanol but not other solvents. With Fura-2/AM, there was an initial two fold increase in fluorescence during the first four hours in methanol and glass with no change in retention time, peak shape or ultraviolet absorption. With Mag-fura, no increase in fluorescence was observed but instead there was a reduction of more than 90% in both fluorescence and ultraviolet absorption within 30 min, indicating that significant decomposition occurs under these conditions. These results confirm previous studies which suggest that acetonitrile is preferable to methanol for sample preparation and chromatographic analysis of Fura-2 and Mag-Fura. These data also indicate that glass vials contain an extractable substance which markedly enhances the spectral intensities of Fura-2/AM.

## 1. Introduction

Fluorescent dye indicators have provided a means of quantitating intracellular free ion concentrations for several cations which are known to play important roles in cell signaling [1,2]. Two of the most significant cations in this respect are calcium and magnesium. The measurement of intracellular free calcium involves introduction into the cell of a fluorescent dye which exhibits different fluorescent spectra in the presence and absence of calcium [3]. The fluorescent properties of Fura-2 have been widely used to estimate intracellular free calcium ion concentrations [1,2]. Fura-2 acid exhibits a maximum fluorescence at 360 nm when not bound to calcium ions. In the presence of calcium ions, fluorescence is enhanced and the fluorescence maximum shifts to 340 nm. The ratio of the fluorescence intensity at these two wavelengths is used as an estimate of changes in the intracellular free calcium ion concentration. Since Fura-2 is polar and thus unable to cross biological membranes, an esterified form, the pentaacetox-

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ymethyl ester (Fura-2/AM), is required to introduce the ion indicator into cells. Once inside the cell, Fura-2/AM is converted by cellular esterases to Fura-2 acid which becomes trapped inside the cell and combines with free calcium ions. Fura-2/AM does not bind calcium ions and has a different fluorescence maximum than does Fura-2 acid.

There are few studies which have addressed the rate of conversion of the ester to the acid or the concentrations of the ester and acid within the cell. One such study used HPLC with fluorescence detection to separate and quantitate Fura-2 acid and Fura-2/AM [4]. These investigators were able to estimate the rate of hydrolysis of the ester in several cell types after extraction with organic solvents. The authors noted that extraction of cells with methanol resulted in significant breakdown of Fura-2/AM and produced numerous peaks with fluorescent properties. No changes in spectral intensities were observed when Fura-2/AM was extracted from cells with acetonitrile.

In preparation for further studies to estimate the intracellular concentrations of Fura-2/AM and Fura-2 acid, we have investigated the stability of Fura-2 in selected organic and inorganic solvents. In the present studies, we have used HPLC coupled to both fluorescence and ultraviolet detection to compare the effects of methanol and acetonitrile on changes in the spectral intensities of Fura-2/AM and a similar ion indicator Mag-Fura AM which is used to estimate intracellular concentrations of free magnesium ions. We have also compared the rates of change of spectral intensities of these ion indicators in glass containers with plastic containers.

## 2. Experimental

Fura-2/AM, Fura-2 acid and Mag-fura AM were obtained from Molecular Probes (Eugene, OR, USA). Stock solutions of each were prepared in dimethyl sulfoxide and stored in plastic vials at −10°C. Glass vials were 4-ml Wheaton (Millville, NJ, USA) borosilicate screw-capped sample vials (No. 224882). Plastic vials were 2-ml Sarstedt (Newton, NC, USA) screw-capped polypropylene micro centrifuge tubes. Methanol and acetonitrile were Burdick and Jackson (Muskegon, MI, USA) high-purity solvents.

Reactions were initiated by adding 20 $\mu$l of the stock solution of the ester (either Fura-2/AM or Mag-Fura AM) to 180 $\mu$l of solvent (methanol, acetonitrile, absolute ethanol, dimethyl sulfoxide or distilled water). The vials were capped and allowed to sit at room temperature. At the designated time period, an aliquot (either 5 $\mu$l or 10 $\mu$l) was drawn directly into a syringe for injection onto the HPLC column.

The HPLC system consisted of a EM Science-Hitachi LiChroGraph Model L-6200 gradient pump, a Varian Model 2050 variable-wavelength ultraviolet spectrometer and a Varian Model 2070 spectrofluorometer. The UV and fluorometric detectors were connected in tandem so that the eluent from the column passed through the UV detector and then immediately through the fluorometer. Chromatographic data, collected on a customized computer data acquisition system, were analyzed by computing both the peak height and the area under the curve for each peak.

A randomized complete block analysis of variance was used to identify difference among groups and a Tukey-Kramer [5] multiple comparison test was performed on all possible combinations to determine significant differences ($p < 0.05$).

## 3. Results and discussion

Previous studies have shown that Fura-2/AM is unstable in methanol but is very stable in acetonitrile [4]. We undertook studies to further characterize these changes which occur with methanol, especially with regard to the rate of decomposition and the breakdown products formed. Based upon previous studies [4,6], we had expected to observe a decline in the fluorescent properties of the esters as they underwent decomposition when exposed to methanol. Instead, we noted a two fold increase in fluorescence within 1 to 2 h of mixing the esters with

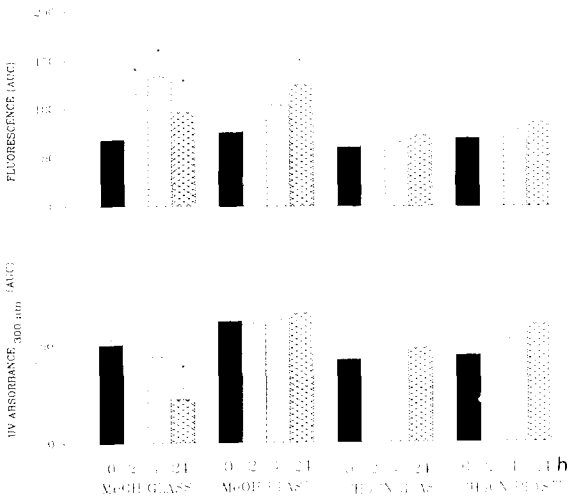

Fig. 1. Changes in spectral intensities of Fura-2/AM in different solvents and different containers. The eluate from the HPLC was monitored by fluorescence detection with excitation at 340 nm and emission at 500 nm and by UV detection at 300 nm. The data represent the mean ± S.D. ($n = 6$–10). The asterisk indicates significant differences ($p < 0.05$) from time zero. AUC = area under the curve.

methanol (Fig. 1). Acetonitrile did not change the fluorescence properties of the ester, even after 24 h of contact. This increased fluorescence with methanol was not accompanied by a change in retention time or in the shape of the ester peak. Although it is unlikely, it is possible that the presence of methanol changes the structure of the compound without altering the retention time. Thus, these changes in fluorescence which occur with methanol appear to represent a change in the fluorescent properties of Fura-2/AM rather than decomposition.

The enhanced fluorescence observed with Fura-2/AM in the presence of methanol is more pronounced when the Fura-2/AM is in contact with glass compared to plastic. The chromatograms illustrated in Fig. 2 indicate that the enhanced fluorescence was not accompanied by a change in retention time or in the shape of the Fura-2/AM peak. An indicated above, these changes in fluorescence were not accompanied by changes in UV absorbency. Acetonitrile had no effect on either the fluorescence or the ultraviolet absorbency of Fura-2/AM, even after 24 h in contact with glass (Fig. 1). As indicated in Fig. 3, the height and shape of the peak remained consistent over 24 h for both fluorescence and ultraviolet absorbency when Fura-2/AM was dissolved in acetonitrile.

A number of studies were performed in an attempt to further characterize the changes caused by methanol and by glass. One possible cause of the increased fluorescence which we considered was initial binding of the ester to glass with subsequent release over a period of time. Our results suggest that this was not the case. Methanol (without Fura-2/AM), when added to glass vials for 10 min and then transferred to a plastic vial containing Fura-2/AM produced a pattern of changes in spectral intensities similar to that observed when Fura-2/AM was added directly to glass (Fig. 4). Fluorescence increased more than two fold by 2 h while no changes were observed in ultraviolet absorbency, even after 24 h. Similar changes were observed when Fura-2/AM was added to a glass vial and immediately transferred to another glass vial (Fig. 4). When glass vials were prewashed with methanol prior to the addition of Fura-2/AM, there were no changes observed in either the fluorescence or the ultraviolet properties of the fluorescent dye. To further prove that adsorption was not occurring, we connected an ultraviolet detector immediately before or immediately after the fluorescence detector. Ultraviolet absorbency did not change in the ester peak during the first 4 h despite a two fold increase in fluorescence. Finally, we were able to demonstrate similar changes in fluorescence occur when a wavelength of excitation of 360 nm was used rather than 340 nm (data not shown).

Fura-2 acid appears to be more stable in methanol than is the ester and is unaffected by glass (Fig. 5). However, there were changes in fluorescence when methanol was transferred from glass to plastic and in ultraviolet absorption when Fura-2 was dissolved in water in a glass container.

Mag-fura exhibited a much different pattern of changes. When Mag-fura was dissolved in methanol in glass vials, rather than an increase in fluorescence as seen with Fura-2/AM, there was a rapid decline with more than 90% of the

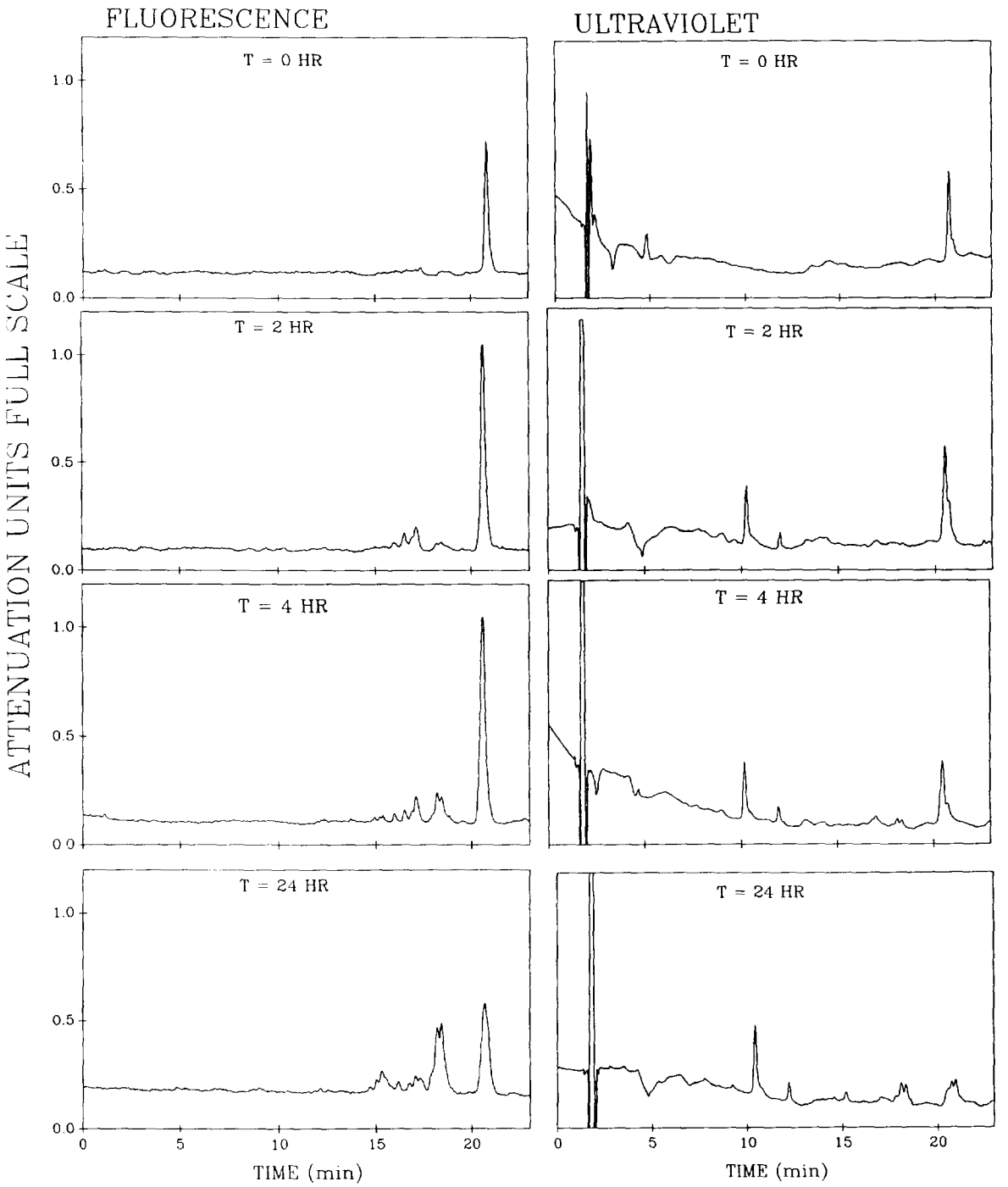


Fig. 2. HPLC of Fura-2/AM at various times after dissolving in methanol in glass vials. The column was a Phenomenex (Torrance, CA, USA) Ultramex $C_{18}$, 250 × 4.6 mm, containing 5 $\mu M$ packing material. The mobile phase (1.5 ml/min) consisted of a linear gradient (3.33% per min) from 20 to 70% acetonitrile in buffer (10 m$M$ $NaH_2PO_4$, 5 m$M$ tetrabutylammonium hydroxide and 0.1 m$M$ $CaCl_2$, pH 5.3). The eluate from the HPLC was monitored by fluorescence detection with excitation at 340 nm and emission at 500 nm and by ultraviolet detection at 300 nm.

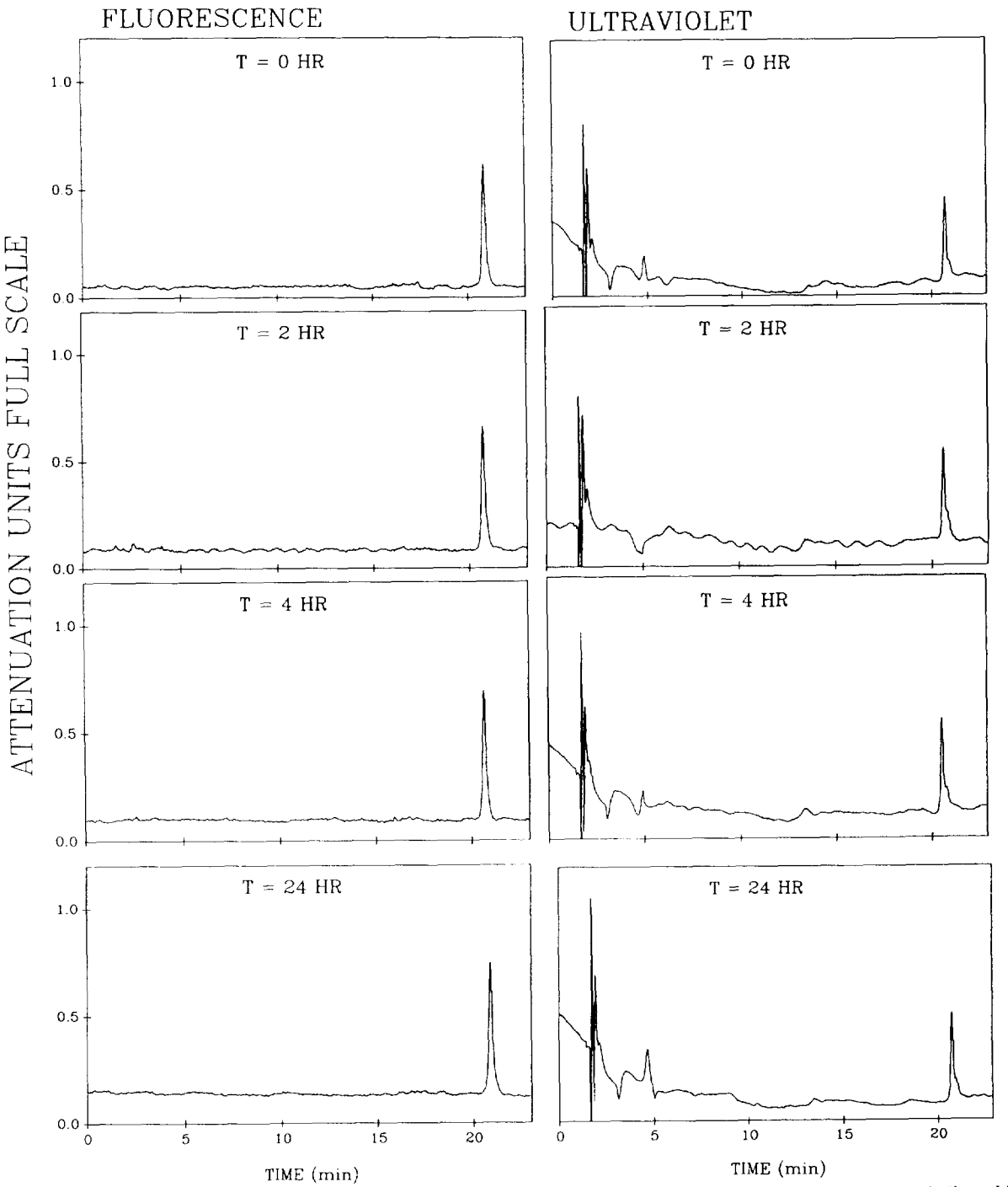


Fig. 3. HPLC of Fura-2/AM at various times after dissolving in acetonitrile in glass vials. Conditions were the same as indicated in Fig. 2. The eluate from the HPLC was monitored by fluorescence detection with excitation at 340 nm and emission at 500 nm and by ultraviolet detection at 300 nm.

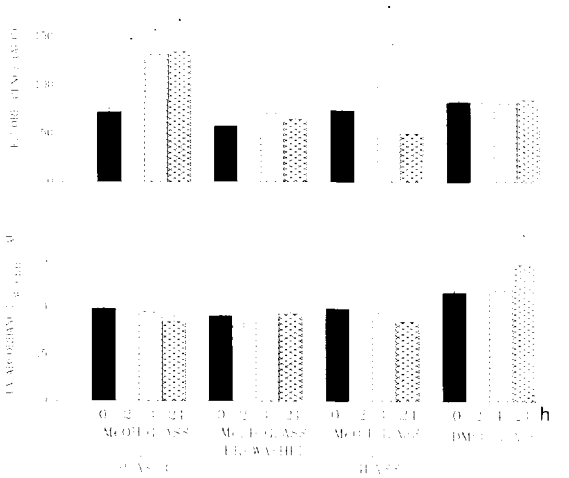

Fig. 4. Changes in spectral intensities of Fura-2/AM in different solvents and different containers. The eluate from the HPLC was monitored by fluorescence detection with excitation at 340 nm and emission at 500 nm and by ultraviolet detection at 300 nm. The data represent the mean ± S.D. ($n = 6$–10). The asterisk indicates significant differences ($p < 0.05$) from time zero. DMSO = dimethylsulfoxide.

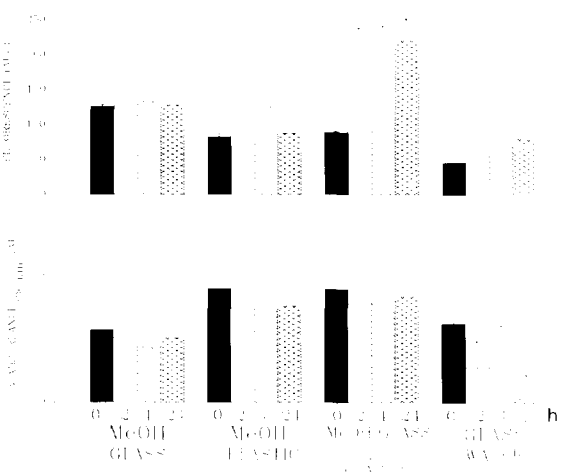

Fig. 5. Changes in spectral intensities of Fura-2 acid in different solvents and different containers. The eluate from the HPLC was monitored by fluorescence detection with excitation at 340 nm and emission at 500 nm and by ultraviolet detection at 300 nm. The data represent the mean ± S.D. ($n = 6$–10). The asterisk indicates significant differences ($p < 0.05$) from time zero.

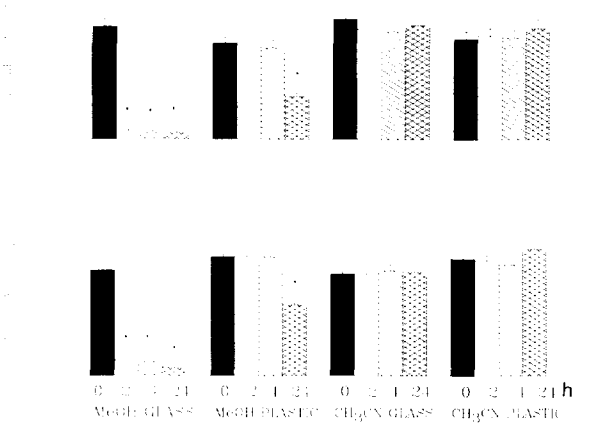

Fig. 6. Decomposition of Mag-Fura in different solvents and different containers. The eluate from the HPLC was monitored by fluorescence detection with excitation at 340 nm and emission at 500 nm and by ultraviolet detection at 300 nm. The data represent the mean ± S.D. ($n = 6$–10). The asterisk indicates significant differences ($p < 0.05$) from time zero.

fluorescence lost within 2 h (Fig. 6). Ultraviolet absorbency followed a similar pattern (Fig. 6). Most of the decomposition which occurred when

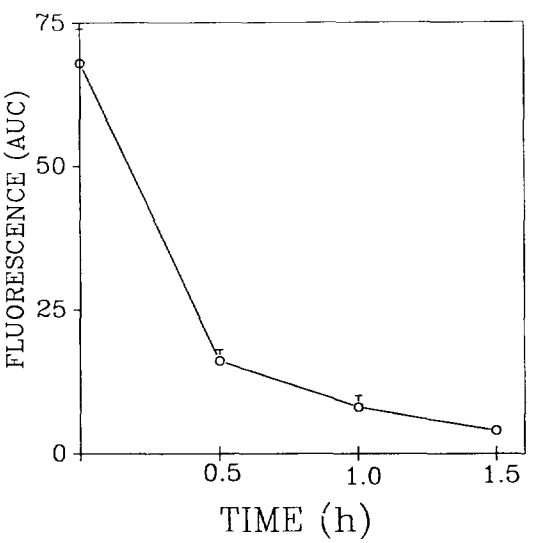


Fig. 7. Decomposition of Mag-Fura dissolved in methanol in glass vials. The eluate from the HPLC was monitored by fluorescence detection with excitation at 340 nm and emission at 500 nm and by ultraviolet detection at 300 nm. The data represent the mean ± S.D. ($n = 6$–10).

Mag-Fura was dissolved in methanol in glass vials occurred within the first 30 min after mixing (Fig. 7). When Mag-fura was dissolved in methanol in a plastic vial no changes were observed over the first 4 h and approximately 50% of the initial fluorescence and ultraviolet absorption remained after 24 h (Fig. 6). Mag-fura was more stable in acetonitrile with no evidence of decomposition during the first 4 h in either glass or plastic containers.

These results confirm previous studies which suggest that acetonitrile is preferable to methanol for sample preparation and chromatographic analysis of Fura-2 and Mag-Fura. Our data also indicate that glass vials contain an extractable substance which markedly enhances the spectral intensities of Fura-2/AM.

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