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Note

Separation of N-(4-aminobenzoyl)-γ-oligo(L-glutamic acid)s on a thin-layer plate and their quantitation using fluorescamine

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Natural folates[1–5] and synthetic antifolates that have demonstrated antitumour activity in clinical trials[6] are converted intracellularly to the γ-oligoglutamates with various numbers of glutamic residues, in which tetra-, penta- and hexaglumates dominate[3,4]. The γ-oligoglutamate chain length, amoung other factors, determines the biochemical activity of folates and antifolates[1,3–6] and many aspects of this relationship remain to be established. Therefore, it is desirable to know the number of glutamic residues in a peptide chain and to quantitate the γ-oligoglutamic derivative(s). For folates, the main method for this purpose is based on the separation of N-(4-aminobenzoyl)-γ-oligo(L-glutamic acid)s (pABGlu$_n$s) resulting from cleavage of the C-9–N-10 bond of folyloligoglutamates. pABGlu$_n$s can be separated by means of liquid column chromatographic methods of various types, including high-performance liquid chromatography (HPLC)[2,5,7,8]. For the detection and quantitation of pABGlu$_n$, fluorescamine appears to be very useful[8].

In this paper, we propose the use of thin-layer chromatography (TLC) to separate pABGlu$_n$s and an adaptation of fluorescamine detection[8] for their quantitation.

EXPERIMENTAL

Materials

Synthetic pABGlu$_n$s (n = 2–6) were obtained as described previously[9]. pABGlu$_1$, pAB and fluorescamine were purchased from Sigma (St. Louis, MO, U.S.A.), Reachim (Moscow, U.S.S.R.) and Roche Diagnostics, Division of Hoffman-La Roche (Nutley, NJ, U.S.A.), respectively. Acetone was dried over phosphorus pentaoxide. Other solvents and triethylamine were of analytical-reagent grade. Chromatography was performed on DC Alufolien 0.25 Kieselgel 60 silica gel plates (E. Merck, Darmstadt, F.R.G.; No. 5553) in a buffer solvent system consisting of methanol–25% ammonia–acetic acid (15:2:1).

TLC separation of pABGlu$_n$s and their detection

Samples of aqueous solutions (3 μl) representing amounts from 30 to 250 pmol

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of each $pABGlu_n$(n = 0–6) were spotted in alternate lanes, 2 cm up from the bottom of a 13 cm layer, using a Hamilton 10-μl syringe. After drying, the plate was developed at 20°C in a solvent-saturated, paper-lined rectangular TLC tank to within 1 cm of the top of the plate. After development, the layer was dried for 1 h using a hot air blast from a hair dryer, sprayed with 1% triethylamine in acetone and then, without drying, with 0.01% fluorescamine in acetone, and finally dried for 5 min at room temperature using a cold blast from a hair dryer. The plate was sprayed again with the same triethylamine solution, dried for a further 5 min at room temperature as above and, after 10 min, viewed under longwave (366 nm) ultraviolet light.

Elution of the $pABGlu_n$–fluorescamine adduct from the thin-layer plate

Each spot, marked under UV light, was cut out. The piece of the plate containing the separated $pABGlu_n$–fluorescamine adduct was placed in a tube containing 1.5 ml of potassium phosphate buffer (pH 7.0) and the tube was shaken vigorously with a microshaker and centrifuged. The elution procedure was repeated. The two supernatants were pooled and completed with potassium phosphate buffer (pH 7.0) to 3 ml. To achieve full recovery of the $pABGlu_n$–fluorescamine adduct from the thin-layer plate, the appropriate elution time and the optimal ionic strength of the buffer used for the elution were investigated.

Fluorescence measurements

To an aqueous solution (5 μl) representing amounts from 25 to 250 pmol of each $pABGlu_n$, placed in a 3-ml cuvette, were added 15 μl of fluorescamine solution (3 mg/ml in dry acetone). The sample was vigorously shaken and, after 1 min, diluted with potassium phosphate buffer (pH 7.0) to 3 ml. Fluorescence was determined using a Perkin-Elmer fluorescence spectrophotometer, either a Model MPF-3L with a sensitivity of 30 or a Model LS-5B, and, according to Furness and Loewen[8], an excitation wavelength of 400 nm and an emission wavelength of 490 nm.

RESULTS AND DISCUSSION

TLC separation of $pABGlu_n$s and their detection

The R_F values of individual sequential $pABGlu_n$s are as follows: pAB 0.81, $pABGlu_1$ 0.63, $pABGlu_2$ 0.50, $pABGlu_3$ 0.39, $pABGlu_4$ 0.29, $pABGlu_5$ 0.21 and $pABGlu_6$ 0.15. The technique of staining with fluorescamine is our own modification of methods proposed in the literature[10–12]. In this instance as little as 30 pmol of each separated $pABGlu_n$ can be detected.

Conditions for the elution of the $pABGlu_n$–fluorescamine adduct from the thin-layer plate

It was found that two elutions each lasting not less than 15 min were necessary to elute the $pABGlu_{1-6}$–fluorescamine adducts from the thin-layer plate in yields above 90%. In our hands the TLC method was faster than the attempted HPLC method[13] because the latter requires a longer time for single sample separation.

The recovery of $pABGlu_n$–fluorescamine adducts eluted from the thin-layer plate was greatly dependent on the ionic strength of the potassium phosphate buffer (pH 7.0). An although 1 *M* buffer was sufficient for almost complete elution of de-

rivatives with up to three glutamate residues, it was necessary to dissolve potassium chloride in 1 *M* buffer at a concentration up to 1 *M* in order to achieve the elution of $pABGlu_{4-6}$. Under these conditions, the recovery of the eluted $pABGlu_{1-6}$–fluorescamine adducts was above 90%.

Fluorescence measurements

Excitation and emission spectra of all $pABGlu_n$–fluorescamine adducts were measured. For all derivatives, the excitation and emission maxima were the same (400 and 490 nm, respectively). The relative fluorescence values of the $pABGlu_n$–fluorescamine adducts (n = 1–6) were measured on a Perkin-Elmer LS-5B instrument using a fixed scale with a factor of 0.1. The values varied around 90 ± 5 and appeared to be the same for all the adducts. The results clearly indicate that the chain length of the oligoglutamates does not affect the relative fluorescence of the $pABGlu_n$–fluorescamine adducts. The dependence of the relative fluorescence on $pABGlu_n$ (n = 1–6) concentration was studied using OBEY Program No. 4 (Perkin-Elmer). Very similar fitnesses were obtained for the fluorescamine adducts formed directly in a tube (correlation coefficient $0.997 < r < 1.0$) and for those formed on a thin-layer plate and subsequently eluted ($0.983 < r < 0.995$). In the former instance, the addition of triethylamine as a stabilizer, needed just for the detection on a thin-layer plate, is not necessary.

CONCLUSION

In our opinion, the method described here can be used for $pABGlu_{1-6}$ quantitation. Furness and Loewen[8] have previously shown that $pABGlu_n$, formed during the reductive cleavage of the C-9–N-10 bond of folylglutamates present in crude extracts from various biological sources, could be quantitated with fluorescamine in the eluate from a DEAE-Sephadex column. In the proposed method, the adaptation of TLC could shorten the analysis time and make it much cheaper. The proposed method can also be applied to the assay of γ-glutamyl hydrolases, which catalyse the hydrolysis of natural folyloligoglutamates[5], in addition to $pABGlu_n$[14]. We have used it to investigate the hydrolase in rat and human serum with $pABGlu_n$ as substrates and obtained enzyme activities[15] comparable to those given by other workers[16,17].

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