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Journal of Chromatography B, 667 (1995) 185–187

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Technical note

Degradation of dansyl polyamines on high-performance thin-layer chromatographic plates

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First received 9 November 1994; revised manuscript received 27 December 1994; accepted 27 December 1994

Abstract

Using high-performance thin-layer chromatography with in situ quantitation to measure dansylated polyamines in the range of 1–20 pmol, we found that dansylated polyamines apparently react with the silica gel of the plates. The fluorescence of the dansyl polyamines diminished with increase in the time interval between application of a sample to the plate and start of the chromatographic separation. Conversely, the fluorescence at the site of application increased with the length of the time interval, indicating the formation of polar reaction products. If this reaction is not accounted for, considerable errors in quantitation of dansyl polyamines may occur.

1. Introduction

Naturally occurring polyamines are being discussed as growth stimulators [1] and as modulators of mitochondrial Ca^{2+} transport [2]. For analysis of polyamines, HPLC is the preferred technique [3], however, the separation of derivatized polyamines by TLC, nowadays often in the form of high-performance thin-layer chromatography (HPTLC), is still widely used [1,4]. Even in very recent literature [1] the time-consuming procedure of scraping marked zones from the TLC plate and measuring the fluorescence of the dansylated polyamines in a fluorimeter cuvette can be found, since virtually no dedicated instrumentation is required. Using HPTLC separation of polyamines derivatized with dansyl chloride (5-[dimethylamino]-naphtalene-1-sulfonylchloride) and in situ quantitation of the fluorescent compounds in the picomolar range, we found that there were apparently irregular losses of fluorescence of the separated polyamines concomitant with an increase in the fluorescence on the site of application.

2. Experimental

Polyamines, gentamicin C sulfate and dansyl chloride were more than 99% pure and obtained from Fluka (Buchs, Switzerland). Solvents were of analytical grade or for-residue-analysis grade from E. Merck (Darmstadt, Germany) or purissimum grade from Fluka. HPTLC plates (silica gel 60, 10×20 cm) and all other reagents of analytical grade were from E. Merck.

The derivatization of polyamines into their respective dansyl derivatives was performed ac-

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0378-4347/95/$09.50
SSDI 0378-4347(94)00596-6

cording to Ref. [5], except for incubation temperature and time which were 56°C and 15 min, respectively. Dansyl polyamines were extracted by vigorous mixing with 2500 μl of toluol. Samples were applied to the plate by spotting (2 × 5 μl) using a Camag Nanomat or by streaking (10 μl, 8 × 1 mm bands) at 10 mm using a self-made apparatus based on a motor-driven Hamilton microsyringe. A stream of nitrogen was directed to the site of application to minimize diffusion of the sample solution. Plates were developed in a one-dimensional multiple development mode in a Camag twin-trough chamber without presaturation or paper lining at room temperature. Eluent A was dichloromethane–triethylamine (80:20, v/v) for development up to 30 mm, eluent B was dichloromethane–toluene–triethylamine (50:40:10, v/v) for development up to 60 mm, eluent C was toluene, for development up to 90 mm. Drying between developments was performed in a vacuum chamber. After separation, the fluorescence was enhanced and stabilized by immersing the plates for 5 s in paraffin–hexane (37:100, v/v) in a self-made dipping apparatus. The plates were scanned in a Desaga CD60 densitometer, using fluorescence excitation at 310 nm and a 420-nm long-pass filter.

3. Results

The limit of determination after fluorescence enhancement was less than 1 pmol, the calibration curve was linear over 2 orders of magnitude. However, the sensitivity was considerably lower when plates were not immediately developed after application of the samples. It was therefore of interest to check whether the fluorescence also showed significant differences between the lanes of a single plate. By repetitive application of the same amount of standard polyamines (12 pmol) at different time intervals (30 s and 2 min, resulting in total application times per plate of 8 min and 32 min, respectively) it became clear, that not only the time interval between application and development was of importance, but also that the fluorescence

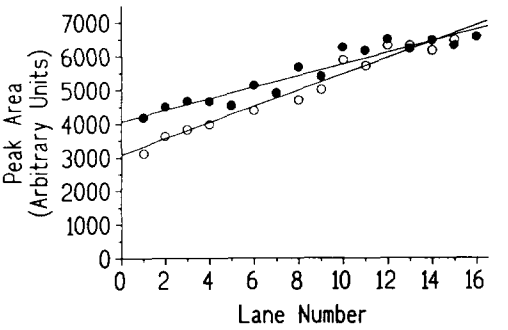

Fig. 1. Decrease of fluorescence of dansyl spermine during application by spotting (2 × 5 μl); (●) 30-s intervals between applications; (○) 2-min intervals between application. Linear regression gave r values of 0.95 and 0.98, respectively.

of dansyl polyamines diminished already during the application process (Figs. 1 and 2). This was particularly impressive when the streaking mode of application was employed (Fig. 2). In the case of streaking at 2 min intervals a nearly complete loss of fluorescence of the first 10 samples resulted (Fig. 2). In the other modes of application, the loss of fluorescence was linear with time. The fluorescence intensity of spot-applied samples was markedly higher than the fluorescence of samples that had been streaked. While this is easily explained by the fact that spotting of the toluene-dissolved samples yielded a smaller starting area than streaking 8 × 1 mm bands, it was surprising to find that also the time-dependent loss of fluorescence during application was

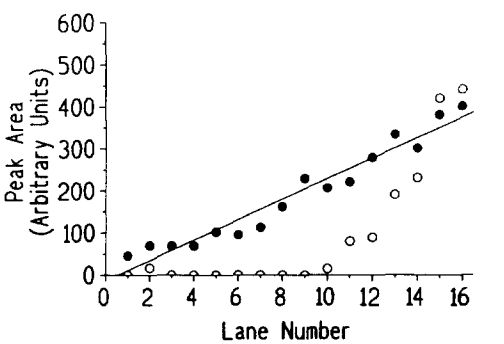

Fig. 2. Decrease of fluorescence of dansyl spermine during application by streaking (10 μl, 8 × 1 mm bands); (●) 30-s intervals between applications. Linear regression gave an r value of 0.97. (○) 2-min intervals between applications.

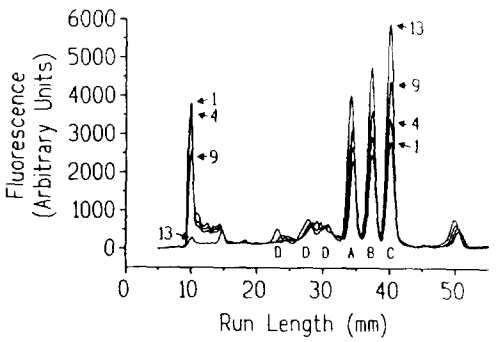

Fig. 3. Decrease of fluorescence intensity of dansylated putrescine (A), spermidine (B), spermine (C), and a gentamicin C mixture (D) and concomitant increase of fluorescence intensity of the starting spot; 10-μl aliquots of a mixture of derivatized standard compounds were spotted in 2-min intervals to an HPTLC plate. Shown are the chromatograms of lanes 1, 4, 9, and 13, corresponding to 32, 26, 16, and 8 min reaction time, respectively.

less severe. In both cases (spotting or streaking) the loss of compound-specific fluorescence was accompanied by an increase of fluorescence at the site of application (Fig. 3). During development, there was apparently no time-dependent loss of fluorescence (data not shown).

4. Discussion

The determination of dansylated polyamines by HPTLC and in situ fluorimetry after fluorescence enhancement is highly sensitive, nearly equivalent to HPLC methods. The resolving power is adequate for many experimental requirements. However, these features can be seriously compromized by the time-dependent loss of fluorescence between the application of the first sample and the start of development. The time-dependent increase of fluorescence at the site of application indicates that the loss of fluorescence is not due to an unspecific fluorescence quenching, but to a reaction of the dansyl polyamines yielding polar reaction products. Since the area of application is related to the velocity of reaction it can be hypothesized that the silica gel of HPTLC plates serves as reaction partner or as catalyst of the degradation. This phenomenon has not yet been described, either because the conventional TLC layer is less reactive, or more probably, because larger amounts of dansylated polyamines were separated and the losses were relatively smaller (lower limit of determination was 25 pmol in Ref. [4] and 15 pmol in Ref. [1]). To minimize the degradation, small sample volumes (1 or 2 μl) should be used to achieve rapid application. When significant losses of fluorescence persist, spotting at defined time intervals and loading the first and the last lane of each plate with the same amount of standard compounds permit the normalization of the fluorescence data before the mass in pmol is calculated.

Acknowledgement

This study was supported by the Deutsche Forschungsgemeinschaft, SFB 330 "Organprotektion".

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