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Short Communication

Standardization in the determination of red blood cell polyamines by high-performance liquid chromatography

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ABSTRACT

The choice of the standardization method in the high-performance liquid chromatographic determination of dansyl polyamines (spermidine and spermine) in red blood cell extracts is discussed. 1,6-Hexanediamine, commonly used as an internal standard, is unsuitable for the quantification of spermidine and spermine in red blood cells because their percentage recoveries are significantly different (100% for 1,6-hexanediamine, and 70% for spermidine and spermine). The external standard method and the standard addition method are better suited. The procedure for the preparation of the standard mixture before dansylation has an influence on the values of red blood cell polyamines. Two procedures are compared and the corresponding percentages of variation were found to be high for spermidine and spermine. Thus the procedure in which the standard is treated in a strictly similar way as the red blood cells is certainly the most appropriate one for the quantification.

INTRODUCTION

The determination of dansyl-polyamines (spermidine (Spd) and spermine (Spm)) in red blood cell (RBC) extracts is generally performed by high-performance liquid chromatography (HPLC) [1,2]. Good results are generally obtained, but it seems that the standardization used is not always correct and needs more attention.

Several authors used 1,6-hexanediamine (DAH) as the internal standard [3,4] for the quantification of polyamines in RBC because it gives a good percentage recovery (100% in this study). This paper questions the validity of DAH as the internal standard because there is a great difference in the percentage recoveries between DAH (100%), Spd (70%) and Spm (70%). An additional point investigated is the influence of the procedure for the preparation of the standard mixture on the erythrocyte polyamine levels. The results of the study emphazise the importance of a correct standardization to obtain good and reproducible results in the determination of dansyl-polyamines in RBC extracts.

EXPERIMENTAL

Reagents and chemicals

Sodium chloride (0.14 *M*), sodium carbonate, perchloric acid, acetone, benzene, acetonitrile and methanol (LiChrosolv) were of analytical-reagent grade (Merck, Darmstadt, Germany).

Stock standard solutions of spermidine (Spd), spermine (Spm) and 1,6-hexanediamine (DAH) (Sigma, St. Louis, MO, USA) were prepared in 5% perchloric acid and stored at −80°C. Dansyl

chloride (100 mg/ml) was dissolved in acetone and the solution was diluted to 5 mg/ml when required.

Apparatus

The HPLC system consisted of two Model 420 pumps (Kontron) coupled to a high pressure mixer, an autosampler MSI 660 (Kontron) with a 7110 Rheodyne injection valve fitted with a 20-μl loop. Separation was achieved on a Nucleosil ODS column (150 × 4.6 mm I.D., 5 μm) protected by a Brownlee RP 18 guard column (30 × 4.6 I.D., 5 μm).

Gradient elution was performed with two mobile phases A (water) and B (methanol) at a flow-rate of 1.0 ml/min: 5 min, 80% B; 2 min, 80% B to 89% B; 5 min, 89% B to 100% B; 3 min, 100% B to 80% B; 10 min, 80% B.

Detection was accomplished using a spectrofluorimeter (SFM 25, Kontron) at an excitation wavelength of 360 nm, and an emission wavelength of 510 nm. This HPLC system was controlled by a microcomputer data system 450 (Kontron).

Dansylation

To 1 ml of erythrocyte extract or standard mixture, 200 μl of saturated Na_2CO_3 and 200 μl of dansyl chloride (5 ml/ml) were added. Tubes were left in the dark for 12 h. The polyamines were then extracted with benzene (two 1-ml volumes) and an aliquot of 200 μl was evaporated under a nitrogen stream. The dried extract was redissolved in methanol before HPLC injection.

Treatment: standard and red blood cells

The procedure is outlined in Table I.

RESULTS AND DISCUSSION

Two procedures for the preparation of the standard before dansylation have been used (see Table I). The standard mixture was treated in a similar way to RBC (RBC standard) or directly dansylated (Normal standard). The values for RBC polyamines, including DAH, are different depending on the standard used (RBC standard or Normal standard at the same concentrations).

Table II shows the percentages of variation for

TABLE I

PROCEDURES FOR THE PREPARATION OF THE STANDARD

	RBC	RBC standard	Normal standard
(1) Plasma and buffy coat are discarded	Centrifugation for 10 min at 2500 *g*		
(2) Washing	RBC pellet washed three times with 0.14 *M* NaCl		
(3) Hemolysis	1 ml from this suspension + 2 ml distilled water	1 ml 0.14 *M* NaCl + 2 ml distilled water	
(4) Deproteinization	+2 ml $HClO_4$ (10%) (RBC without added) +2 ml $HClO_4$ (10%) with DAH, Spd, Spm (RBC with added). Centrifugation for 10 min at 30 000 *g*	2 ml $HClO_4$ (10%) with DAH, Spd, Spm	Standard mixture (DAH, Spd, Spm) prepared in $HClO_4$ (5%)
(5) Dansylation and extraction		1 ml dansylated extract	

TABLE II

PERCENTAGES OF VARIATION OF THE RBC POLYAMINE VALUES OBTAINED WITH THE RBC STANDARD IN COMPARISON WITH THE NORMAL STANDARD

Polyamine	Percentage of variation (mean ± S.D., n = 10)
DAH	13 ± 2.1 decrease
Spd	24 ± 1.1 decrease
Spm	29 ± 2.2 increase

the results obtained with the RBC standard compared with the Normal standard. The procedure (see Experimental), and consequently the medium in which the standard is prepared has an influence on the results. The only difference in the treatment of the standards is the presence of NaCl (30 m*M*) in the RBC standard compared with the Normal standard. If the dansylated volume and pH are similar, it may be asked whether the ionic strength has an effect on dansylation and thus on the results. However, it seems logical that the RBC standard, which is prepared in a similar way to RBC, is the most appropriate one for the quantification of polyamines in RBC.

The unsuitability of DAH as an internal standard is demonstrated by the method of standard addition. This method, which consists of adding known amounts of DAH, Spd and Spm in perchloric acid (during deproteinization), gives different recoveries (DAH 100%, Spd 70%, and Spm 70%) regardless of the standard used (Normal or RBC). DAH is not a good internal standard (although often cited in the literature) because it tends to underestimate the Spd and Spm levels in erythrocytes. For the other tissues [5,6] or physiological fluids, this problem must be the same since the differences between Spd, Spm and DAH are due to the structures of these molecules. The number and positions of the amine functional groups are two influential parameters during dansylation.

Thus the external standard method and the standard addition method (although technically more tedious) are better suited to the quantification of polyamines in red blood cells and of tissular polyamines in general. These problems of standardization should not be underestimated in the establishment of the polyamine base values. They become less important when relative values are under test, for example during follow-up of a patient.

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