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FEMTOMOLAR ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINING Dns-POLYAMINE DERIVATIVES OF RED BLOOD CELL EXTRACTS UTILIZING AN AUTOMATED POLYAMINE ANALYZER

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SUMMARY

An ultra-sensitive automated method for the determination of polyamines in red blood cell extracts by ion-pair reversed-phase high-performance liquid chromatography is described. The 5-dimethylaminonaphthalene-1-sulfonyl derivatives of putrescine, 1,6-diaminohexane, spermidine, and spermine are separated on a μBondapak C_{18} column using 1-heptanesulfonic acid and acetonitrile as the mobile phase. All compounds are eluted within 28 min subsequent to the initial injection. The method has a lower detection limit of 25 fmoles on column.

Because of the simplicity and ease of operation, the method is applicable for use in either the research or clinical laboratory.

INTRODUCTION

During the past five years, high-performance liquid chromatography (HPLC) has become an approved and accepted technique for use as an exploratory tool for studying various research problems previously restricted to more established and conventional methodologies. As such, its continuous and expanded use has led to the development and improvement of many new and refined methods based on the application of classical analytical procedures[1,2]. The work of Seiler and Wiechmann[3], who used 5-dimethylaminonaphthalene-1-sulfonyl (Dns) chloride as a fluorometric probe to improve upon the sensitivity of polyamines is an excellent example of one of these classical methodologies used by various researchers.

Utilizing many of these new innovations, we describe a HPLC system designed specifically to operate as an automated polyamine analyzer. Coupled with an ultra-sensitive method, the complete system can be used to assay Dns derivatives of putrescine, spermidine, and spermine in various types of biological fluids. The analyzer and method are extremely simple and specific. Systems operations, which include, sample

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injection, gradient programming, and data handling are automatically controlled and monitored by a reiterative preprogrammed scheme.

Operating with a 48-sample-run capacity, the analyzer can be programmed to operate in an automated mode for more than 29 h. Analysis and equilibration require 37 min per sample. Polyamine amounts as low as 100 fmoles on column are quantifiable by the procedure.

Because of the high signal-to-noise (S/N) ratio of the system, its use is completely applicable for measuring extremely low concentrations of polyamines in certain cell culture systems. As such, we are currently utilizing this new analytical system and method in our anti-malarial drug screening program for studying the inhibitory effects of various compounds on the growth cycle of *Plasmodium falciparum* during different stages of intraerythrocytic growth.

EXPERIMENTAL*

Materials

A Waters Model APC/GPC-204 liquid chromatograph was used to complete this study. The analytical system consisted of two Model 6000A high-pressure pumps, a Model 660 solvent programmer, and a Model 710B Waters intelligent sample processor (WISP) auto-injector. An Aminco Fluoromonitor, equipped with a 365 nm excitation and 485 nm emission filter was employed for fluorometric detection. A Houston Instrument, Omniscribe A5000 dual-pen recorder and a Columbia Scientific Industries Supergrator-3 integrator recorded and integrated all peak areas. HPLC-grade spectroquality acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) mixed in a gradient mode with PIC-B7 (1-heptanesulfonic acid, Waters Assoc., Milford, MA, U.S.A.) was employed to separate the various Dns-polyamines. Standard solutions of each polyamine (20 nmoles/ml) were prepared in 0.005 *N* hydrochloric acid by using 99% putrescine hydrochloride, 99% spermidine hydrochloride, 97% spermine hydrochloride, and 1,6-diaminohexane hydrochloride (Aldrich, Milwaukee, WI, U.S.A.). A 2 mg/ml solution of Dns chloride in acetone (Dns chloride purchased as a 100 mg/ml solution in acetone from Pierce, Rockford, IL, U.S.A.) was used to derivatize all samples.

Procedures

Dns-polyamines were prepared as follows: 200 μl of red blood cell (rbc) extracts, as described below, were pipetted into 13 mm × 100 mm screw-capped cultured tubes. An 8-μl volume of a 20 nmoles/ml solution of 1,6-diaminohexane (internal standard), 292 μl of 0.5 *M* carbonate buffer (pH 9.2), 100 mg of anhydrous potassium carbonate, and 500 μl of 2 mg/ml of Dns chloride in acetone were added and thoroughly mixed. The tubes were capped and the samples were allowed to react at room temperature for 16 h, overnight. Upon completion of the derivatization, 5 ml of *n*-heptane were added to each tube and the samples were vortexed for 30 sec. The organic phase, consisting of both acetone and *n*-heptane was approximately 5.5 ml. A 5-ml volume of the organic layer was removed and pipetted into a 13 mm × 100 mm

* The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

culture tube. The solutions were blown to dryness with streams of nitrogen gas in a 30°C water bath. The dried samples were reconstituted with 200 μl of HPLC-grade methanol and 10 μl of glacial acetic acid. Samples can be analyzed immediately, but for better results they were refrigerated for 3 days at 4°C and then analyzed. From previous studies, it has been shown that the derivatized specimens are stable for more than 6 months when capped and stored at −20°C.

A prepacked 30 cm × 3.9 mm I.D. μBondapak C_{18} column was used to chromatograph standard and experimental Dns-polyamines. μBondapak C_{18} is a 10-μm particle size packing material, which is used for both analytical and semi-preparative separations. The mobile phase consisted of 0.02 *M* solution of 1-heptanesulfonic acid combined with acetonitrile. PIC-B7 reagent was prepared by mixing 40 ml of the pre-packaged solution with 460 ml of glass distilled water. The pH of the mixture was 3.40. A concave gradient (Curve 9, solvent programmer) was used to elute the various Dns-polyamines from the column. Curve 9 may be duplicated in any dual-pumping chromatographic system by using the following formulae.

$$\%\text{ flow from pump B} = (FC - IC)\left(\frac{t}{T}\right)^m + IC$$

$$\%\text{ flow from pump A} = 100\% - \%\text{ flow from pump B}$$

where FC = final concentration; IC = initial concentration; t = time into the run; T = time for the total gradient run; and m = 4.00.

Gradient parameters were 50% acetonitrile and 50% 1-heptanesulfonic acid at zero time. Upon injection, the acetonitrile was increased from 50 to 80% within a 22-min period. Total analysis time was 28 min. Flow-rate for the dual pumping system was 2 ml/min. Column pressures ranged between 1200 and 1500 p.s.i. (83–103 bar). Separations were performed at ambient temperatures. The initial standard of 1,6-diaminohexane was used to monitor and to normalize peak areas. Each specimen was run in triplicate to insure reproducibility. Peak areas were measured by an on-line computing integrator. The lower detection limit of the method was 25 fmoles on column with a S/N ratio of 5:1.

Program parameters

The reiterative program scheme, which operated the total system was controlled by the WISP auto-injector. The format for this scheme was programmed as follows:

(a) Initiate automated mode;
(b) Key sample number, key "0", enter;
(c) Key injection volume, key 10 μl, enter;
(d) Key run time, key 28 min, enter;
(e) Key number of injection, key 1, enter;
(f) Key equilibration delay, key 9, enter;
(g) Initiate "run" mode.

Samples

A 100-μl volume of normal or parasitized packed rbc was hemolyzed with 900 μl of distilled water. A 0.4-ml volume of the hemolysate was removed and de-

proteinized with 12% cold perchloric acid. Samples were centrifuged, with the supernatant removed and neutralized with 1 *N* K_2CO_3 solution. The resulting rbc extracts were brought to a 1-ml volume with distilled water, thereby producing a 1:25 dilution ratio to that of the original sample amount. Specimens were then derivatized.

RESULTS AND DISCUSSION

Polyamine synthesis, which is an extremely important biochemical process involved in cellular growth in both prokaryotic and eukaryotic systems has initiated a great deal of research over the past 10 years[4–6]. However, much of this research has focused primarily on polyamines and their relationship to various proliferative disease states. While these investigations have contributed much to this area of study, little information defining the role of polyamines in host–parasite interactions has been made available.

It has been reported that the protozoan parasites of the genera *Plasmodium* and *Trypanosoma* rely heavily on the biosynthesis of polyamines and diamines during their life cycle, and as such, a moderate disruption in this biochemical process, especially during the logarithmic phase, will drastically inhibit the normal growth of these organisms.

In an attempt to understand some of the facets involved in this process, our laboratory is currently utilizing a new analytical polyamine analyzer, along with an *in vitro* cell culture system to study the inhibitory effects of D,L-α-difluoromethylornithine (DFMO) and similar drugs on the growth of *P. falciparum*. DFMO, an experimental drug which acts as an irreversible competitive inhibitor of the enzyme, ornithine decarboxylase (ODC) in parasites, blocks the biosynthesis of putrescine in these organisms (Fig. 1), thereby restricting the synthesis of spermidine

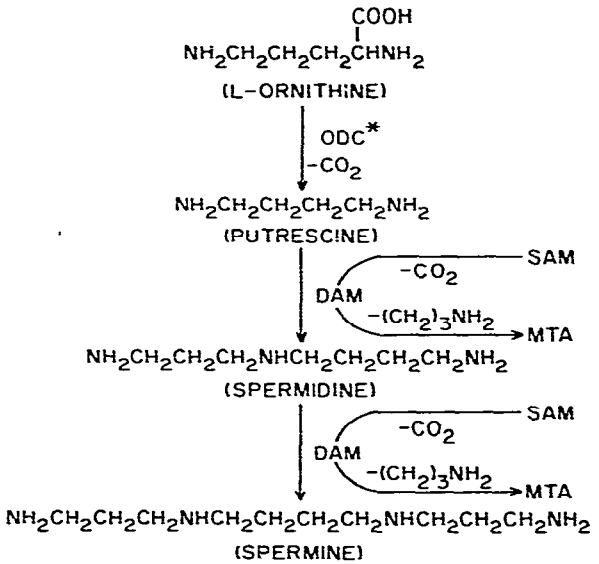


Fig. 1. Biosynthetic scheme showing the formation of putrescine, spermidine, and spermine in eukaryotic systems. ODC* = Ornithine decarboxylase; SAM = S-adenosylmethionine; DAM = decarboxylated adenosylmethionine; MTA = methylthioadenosine. (* = pivotal enzyme).

and spermine. The effects of various ODC inhibitors on parasites in the *in vitro* rbc culture system were demonstrated both morphologically and biochemically. DFMO concentrations of $3 \cdot 10^{-4}$ *M* and greater resulted in retarded growth and decreased polyamine levels, especially putrescine and spermidine. Parasatized cells showed a decreased number of "rings,, (early trophozoites), misshapened "rings", along with a decrease in parasite cytoplasm. The biochemical synthesis of polyamines was similarily affected in the plasmodia, as shown by the analytical data obtained from this study.

From a series of chromatograms shown below, the applicability of the method is demonstrated. Fig. 2 represents the separation of a standard solution, containing 750 fmoles of Dns-putrescine, 1,6-diaminohexane, spermidine, and spermine. Linearity was observed for all concentrations used in this study (100 f*M*–25 p*M*). Correlation coefficients for putrescine, spermidine, and spermine were 0.911, 0.929, and 0.937, respectively.

Because of the ubiquitous nature of polyamines and the pervasiveness of these compounds in the environ, extreme precautions must be taken to prevent sample contamination. Fig. 3, a chromatogram of a glass-distilled water and internal standard sample shows the relative purity of the derivatized sample.

Based on these initial separations, normal rbc extracts were chromatographed to establish the profile for unparasitized specimens (Fig. 4). Putrescine and spermidine were the only polyamines present in a 10-μl injection. However, when the sample volume was tripled, spermine could be detected.

In contrast, analysis of the extract from parasitized rbc produced different results from that of the normal rbc extracts (Fig. 5). All three polyamines were

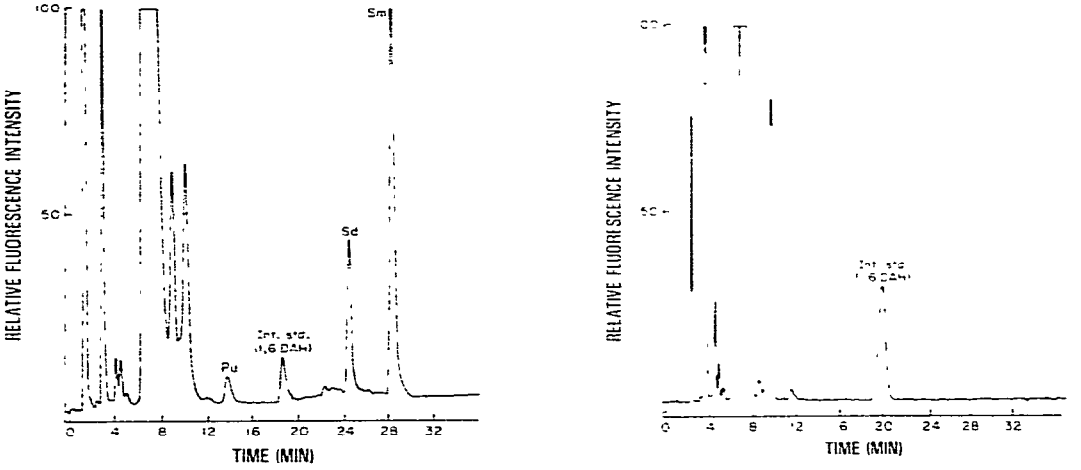
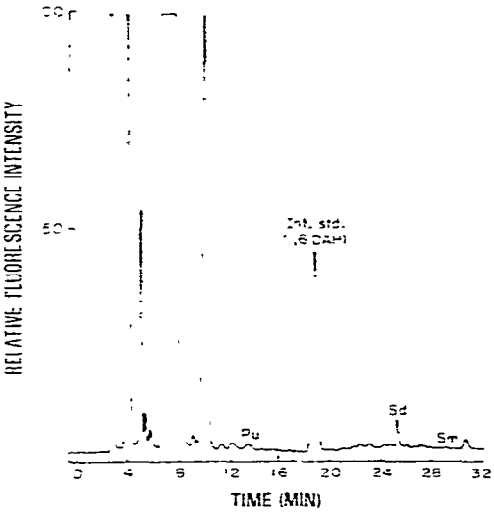


Fig. 2. Separation of a standard solution containing 750 fmoles of putrescine (Pu), spermidine (Sd), and spermine (Sm). Column: 30 cm × 3.9 mm μBondapak C_{18}. Mobile phase: gradient mode 50% acetonitrile–50% 0.02 *M* 1-heptanesulfonic acid (zero time) 50–80% acetonitrile (22 min), 2 ml/min. Column temperature: ambient. Internal standard (Int. std.) is 1,6-diaminohexane (1,6 DAH). Meter multiplier: 0.3.

Fig. 3. Chromatogram of a control blank detected at 365 nm excitation and 485 nm emission. Meter multiplier: 0.3.



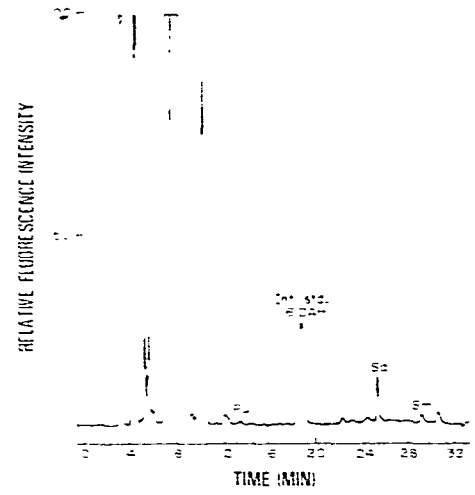


Fig. 4. Separation of a normal red blood cell extract (unparasitized). Sample volume: 30 μl. Meter multiplier: 0.3. Abbreviations as in Fig. 2.

Fig. 5. Chromatogram of a 5% parasitized red blood cell extract. Sample volume: 10 μl. Meter multiplier: 0.3. Abbreviations as in Fig. 2.

present in the parasitized sample. Spermidine and spermine were significantly higher in concentration in these extracts than in their unparasitized counterparts. Putrescine levels were usually equal to or just a little higher, depending upon the stage of parasitemia of the blood cells.

When DFMO was incubated in the cell culture system with parasitized rbc for 24 h at 37°C, a drastic reduction of all three polyamines was observed (Fig. 6).

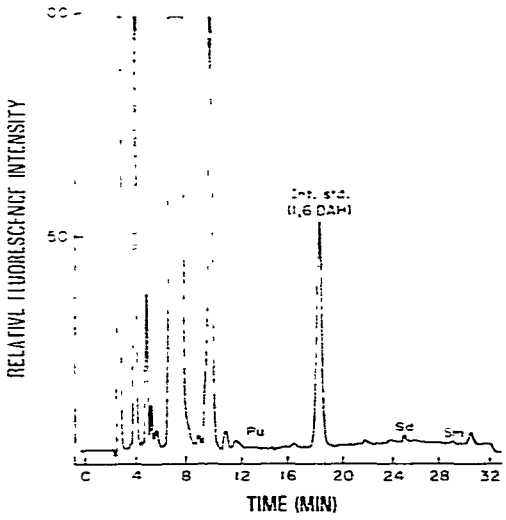


Fig. 6. Chromatogram of a 5% parasitized red blood cell specimen treated with D,L-α-difluoromethylornithine (DFMO) for 24 h at 37°C. Sample volume: 10 μl. Meter multiplier: 0.3. Abbreviations as in Fig. 2.

TABLE I

PRECISION OF AN ION-PAIR HPLC METHOD FOR QUANTIFYING Dns-POLYAMINES IN NORMAL AND PLASMODIAL INFECTED RED BLOOD CELLS

Values given as mean ± standard deviation (n = 8). N.D. = Not detectable; rbc = red blood cells.

	Polyamines detected in rbc (pmoles/ml)		
	Putrescine	*Spermidine*	*Spermine*
Normal rbc	189 ± 4.6	222 ± 6.2	<5
Parasitized rbc	281 ± 7.9	779 ± 19.1	45 ± 1.3
DFMO-treated parasitized rbc	N.D.	110 ± 3.8	9 ± 0.3

N.D. = Not detectable.

Putrescine disappeared completely, while spermidine and spermine were reduced approximately 6-fold. The results shown in Table I summarize the data collected from this study.

Finally, in order to reproduce precise and reliable data, the samples used for the precision of the method were subjected to multiple analyses. The automated polyamine analyzer employed in this study reduced the tediousness and difficulty usually associated with such an undertaking. The ease of operation is demonstrated by the chromatograms shown in Fig. 7. The three samples depicted in these chroma-

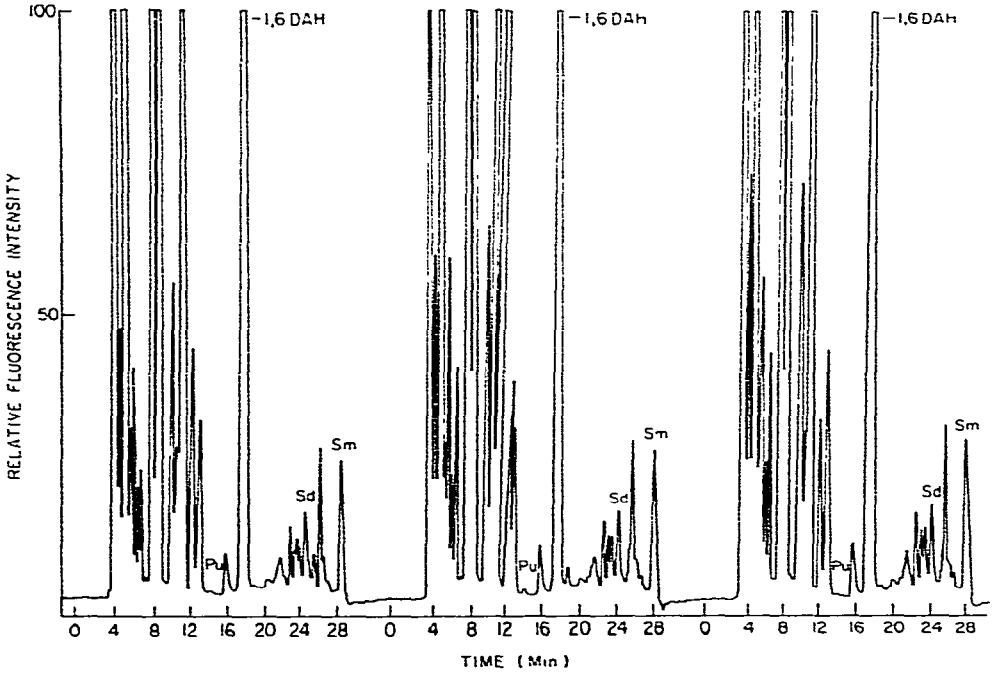


Fig. 7. Separations of three parasitized red blood cell extracts taken from a 48-sample-run, demonstrating the applicability of the polyamine analyzer. Meter multiplier: 0.3.

tograms were taken from a 48-sample-run. Because of the elimination of the human element in analyzing such a large number of samples over an extended period of time, both precision and accuracy are enhanced by such a system.

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