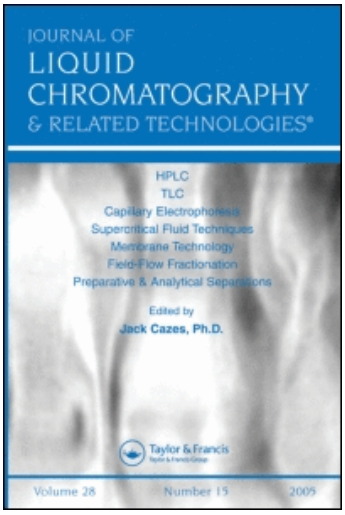

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Analysis of Polyamines and Their Acetylated Forms with 9-Fluorenylmethyl Chloroformate and Reversed Phase HPLC

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ANALYSIS OF POLYAMINES AND THEIR ACETYLATED FORMS WITH 9-FLUORENYLMETHYL CHLOROFORMATE AND REVERSED PHASE HPLC

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ABSTRACT

A simple, fast and sensitive reversed phase HPLC method has been developed for analysis and quantitation of acetylated and non acetylated forms of polyamines. The polyamines are pre-column derivatized with 9-fluorenylmethyl chloroformate (FMOC) and detected in fluorescence. The derivatization reaction is completed in less than two minutes. The excess of FMOC is derivatized with glycine which reduces the interference from the non reacted reagent to a minimum. The conditions favor separation and detection of N^1-acetylputrescine, putrescine, N^8-acetylspermidine and N^1-acetylspermine in a standard of 10 fmol. Spermidine and spermine are detected at 100 amol. Here, the method is used for analysis of extracellular polyamines in synovial fluid from patients with rheumatoid arthritis and for intracellular detection of rabbit lens polyamines.

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INTRODUCTION

The polyamines are normal cell constituents that play an important role in both normal and pathological tissue growth, cellular differentiation and membrane function (1-5). Their involvement in inflammatory stimuli (6-7) and anti inflammatory reactions (8) has been discussed. They also seem to be a part of downregulation of the immune reactivity and are thought to contribute to the interleukin 2 deficiency in rheumatoid arthritis (9).

The principal polyamines in biological systems are putrescine; $H_2N(CH_2)_4NH_2$, spermidine; $H_2N(CH_2)_4NH(CH_2)_3NH_2$ and spermine; $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$. Because of their strongly cationic nature these amines interact with proteins, phospholipids and nucleic acids (4, 10). Polyamines exist in biological material in three states; as a free base, as a conjugate (e.g. monoacetylated) and bound (10-11). Still, a large part of their physiological function remains to be clarified.

In the last few years, the determination of polyamines by pre- or postcolumn derivatization and reversed phase HPLC techniques have been accepted. The interest of the techniques used, have been focused on the methods of derivatizing polyamines with dansyl chloride, ortophthalaldehyde (OPA) (12-18) and more recently, 9-fluorenylmethylchloroformate (FMOC) (19-20) which reacts with both primary and secondary aminogroups (21). By modifying the FMOC-amino acid method of Gustavsson and Betnér (22), we eshtablished improved conditions for the analysis of polyamines in biological samples.

The FMOC derivatized polyamines and the reagent itself have identical excitation and emission spectra. Therefore, excessive reagent has to be removed prior to injection to facilitate quantitation of all the polyamine derivatives. In this study, the removal is attained by derivatizing the non reacted FMOC with glycine. The reproducibility of the derivatization reaction, the quantum yield of the fluorescence at different mobile phase compositions, the stability of derivatized samples and the detection and quantitation of acetylated polyamines

were investigated. The method has been used for analysis of polyamines in human synovial fluid and rabbit eye lense extract.

MATERIALS AND METHODS

Apparatus

The HPLC system (Kabi-Pharmacia LKB Biotechnology, Bromma, Sweden) consisted of two Model 2150 pumps equipped with a high pressure mixer, a Model 2152 LC controller for gradient programming and a Rheodyne injection valve 7125 with a 20 µl sample loop. Fluorescence was monitored with a Shimadzu, Model RF 535 Fluorescence HPLC monitor with a 12 µl flow cell and a xenon lamp (Kyoto, Japan). Measurements were made at an excitation wave-length of 260 nm and an emission wave-length of 315 nm. The chromatograms were recorded with a Model 2210 recorder (Pharmacia LKB). Quantitative evaluations were performed with a Nelson Analytical 3000 Series Chromatography Data System connected to a Nelson 900 Series Interface (Nelson Analytical Inc. California, U.S.A.).

The HPLC column, a SuperPac™ Cartridge (4,0 x 125 mm), packed with 3 µm Spherisorb ODS 2 and a guard column (4,6 x 10 mm) packed with the same material was also obtained from Pharmacia LKB. The column was maintained at room temperature (+21° C).

Mobile Phase

Ultrapure water (MilliQ), exclusively used in all buffer preparations was generated with a Milli Q Water Purification System (Millipore, U.S.A.). This system included Super C, Ion Exchange, Organex Q cartridges and a 0,22 µm MilliPak filter. All buffers were filtered through a 0,45 µm Millipore filter before use. Unless specified, the

chemicals used were of analytical grade and the solvents of chromatographic grade.

Eluent A consisted of 70% 50 mM acetic acid (Suprapur® Merck, Darmstadt, FRG), pH 4,2 and 30% acetonitrile (Super gradient HPLC, LabScan Analytical Sciences, Ireland). The acetate buffer was prepared by adjusting the acetic acid in MilliQ with 30% sodium hydroxide solution (Suprapur®, Merck). Eluent B comprised 100 % acetonitrile. The flow rate was 1,25 ml/minute.

Derivatization

9-Fluorenylmethyl chloroformate (FMOC) (Fluka Chemie, Switzerland) was dissolved in acetonitrile. Borate buffer, 0,5 M, was prepared from boric acid (Merck) and pH was adjusted to 8,5 with sodium hydroxide solution (Suprapur®). Glycine and N-methyl-butylamine (MBA) (Aldrich-Chemie, Steinheim, Germany) were dissolved in acetonitrile:MilliQ (1:1) to a final concentration of 12 mM each. In additional experiments, the FMOC reagents were dissolved in acetone (Merck) instead of acetonitrile.

To 10 µl sample, 10 µl borate buffer and 10 µl FMOC-reagent were pipetted and immediately mixed. After 45 seconds 20 µl glycine or MBA solution was added and vortexed. After another 45 seconds the mixture was ready for injection.

Polyamines

Polyamines were obtained as hydrochloride salts from Sigma, St Louis, MO, U.S.A. Standards of N^1-acetylputrescine, putrescine, spermidine, N^8-acetylspermidine, spermine and N^1-acetylspermine were prepared in concentrations from 0,125 - 25 µM in Milli Q and kept at -20°C until analyzed.

For sample quantitation, 25 - 125 nM of the standards including the internal standard 1,6-diaminohexane (DiHEX), (Sigma) were dissolved in acetonitrile:MilliQ (2:1)

The Fluorescence Response at Different Mobile Phase Compositions

The quantum yield of fluorescence was measured in derivatized samples of PUT, SPD and SPM. Six nmol of each polyamine was derivatized separately as described and diluted 1000 times in acetonitrile:MilliQ. The samples were pumped through the fluorescence monitor and the response at 0, 20, 40, 60, 80 and 100% acetonitrile respectively was measured.

Sample Preparation

Synovial fluid from five rheumatoid arthritis patients were used. Each sample was deproteinized with two volumes acetonitrile, centrifuged (23), derivatized and injected separately. The internal standard DiHex was added prior to deproteinization.

The water-soluble part of an 8 g amount of rabbit eye lenses was dissolved in 8 ml Milli Q. The sample was deproteinized as described above. No additional extraction of the polyamines was done.

RESULTS AND DISCUSSION

Derivatization

The FMOC solvents, acetonitrile and acetone were tested. The fluorescence response as well as the linearity and the reproducibility was better with acetonitrile in the derivatization medium instead of acetone. Therefore, in the results presented, acetonitrile is used.

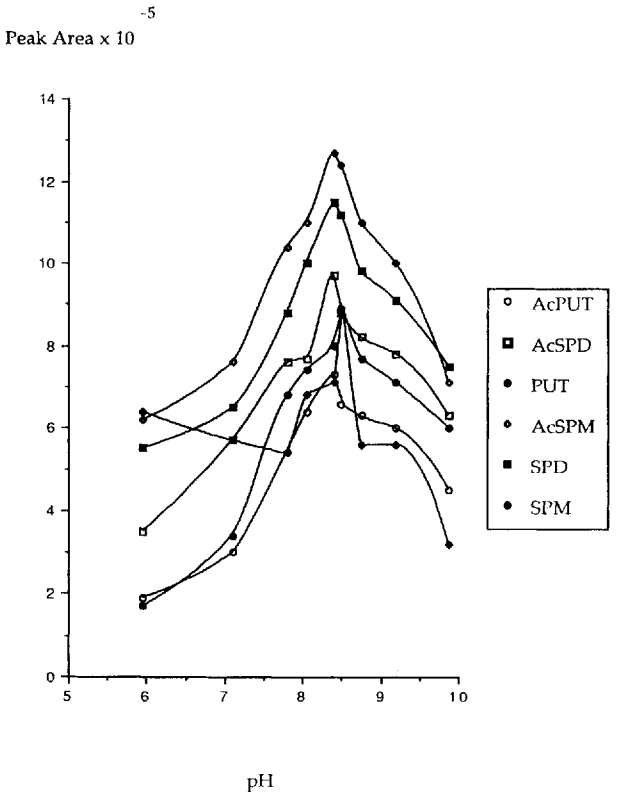

FIGURE 1. Effect of borate buffer pH on peak area. (n =3)

For positive identification of the different polyamines, they were injected on the column one by one. Three separate derivatizations of the standards were done in each experiment

In the FMOC-polyamine reaction, pH was found to be critical, though it controls both the derivatization and the hydrolysis product of FMOC (19-20). Figure 1 shows the peak area, corresponding to fluorescence intensity for each polyamine at various pH. This study shows that the optimum pH was 8,5.

In order to achieve complete derivatization of the polyamines in a sample, it is necessary to use excess FMOC. This was confirmed in an

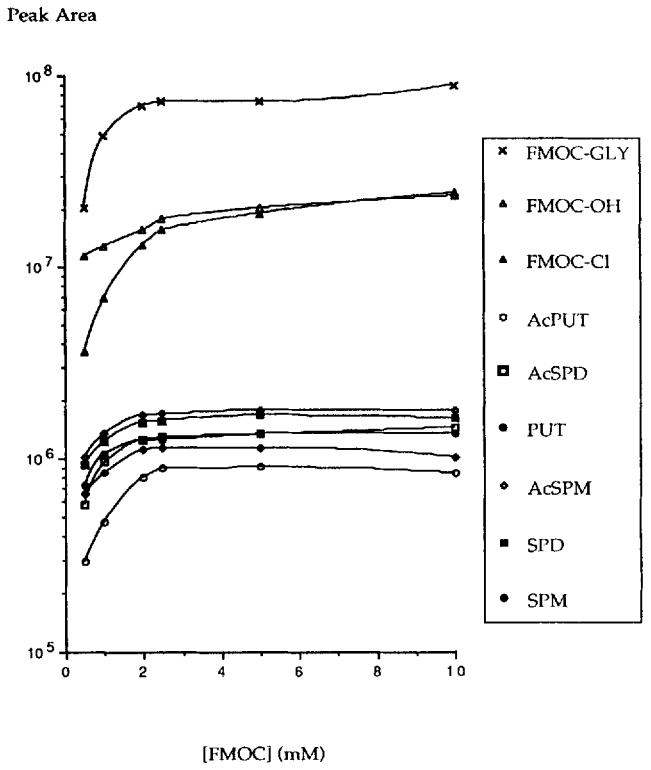

FIGURE 2. Effect of FMOC-reagent concentration on peak area. (n =3)

experiment where the peak area of a polyamine standard (2,5 μM/polyamine) was measured when the concentration of the FMOC-reagent varied from 0,5-10 mM (Fig.2). The peak area response yields a plateau at 2,5 mM FMOC. At higher FMOC concentrations, the increase of the hydrolysis product and the non reacted FMOC interferes with the chromatographic separation.

It is important to remove non reacted FMOC before separation, since the hydrolysis product and other reagent peaks formed, elutes together with some polyamine derivatives (Fig.3). This can be achieved by pentane or diethyl ether extraction (24-26) or derivatization (22, 27).

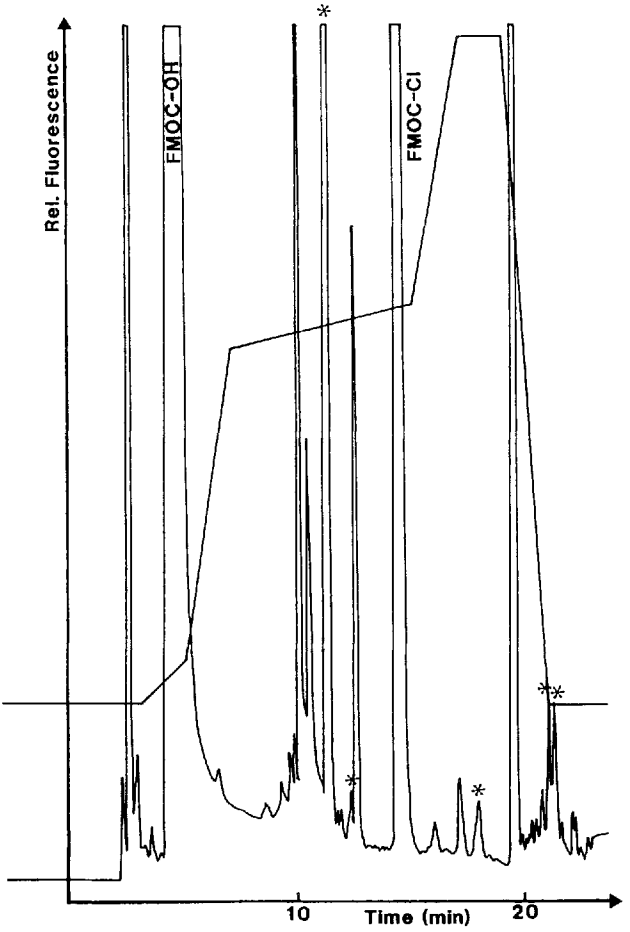

FIGURE 3. Chromatogram of a blank (derivatized MilliQ water). No glycine is added and the excess FMOC products are shown. Peaks that disturbe a polyamine separation are marked (*). Injection volume 20 µl, flow- rate 1,25 ml/min. Gradient: 0-3 min 25% B isocratic, 3-5 min 30% B, 5-7 min 65% B, 7-15 min 70% B, 15-17 min 100% B, 17-19 min 100% B isocratic, 19-21 min 25% B. Fluorescence detector; 1/64, 2 mV.

Pentane extraction of the sample (2x100 µl pentane) transfers about 80% of the hydrolysis product but also more than 90% of the polyamine derivatives into the organic phase. To avoid this problem, the hydrophilic amino acid, glycine, or the highly reactive MBA was added to a blank (derivatized MilliQ water) and derivatized with the excess of FMOC. Under given conditions, the FMOC-glycine derivative will be eluted first in the chromatogram, and disturbance from other FMOC-peaks in a polyamine separation is reduced to a minimum (Fig. 4 and 5). However, FMOC is not completely reacted and approximately 4 % of the reagent remains as FMOC-Cl. Higher concentrations of the acid glycine does not improve the reaction yield because of a decrease in pH. With MBA to remove excess FMOC, the non reacted FMOC-Cl is 2,5 %, but MBA does not separate successfully from putrescine. A lower concentration of MBA increases non reacted FMOC-Cl. When glycine is present, the 4 % of the non reacted FMOC do not disturb the separation. Glycine is therefore chosen for further experiments.

Linearity

The linearity of the FMOC-polyamine derivatives was tested with three separate derivatizations at each concentration. The response was linear ($r > 0,997$) for each polyamine between 0,5-100 pmol (Fig. 6).

Reproducibility

Reproducibility of the peak area was estimated from 10 different derivatizations during three days, of a standard mixture from which 10 pmol of each polyamine was injected. The Relative Standard Deviation (RSD%) varied from 2,4-5,0 (Table 1). The reproducibility of the method could be further improved by using automated injection and automated precolumn derivatization (22).

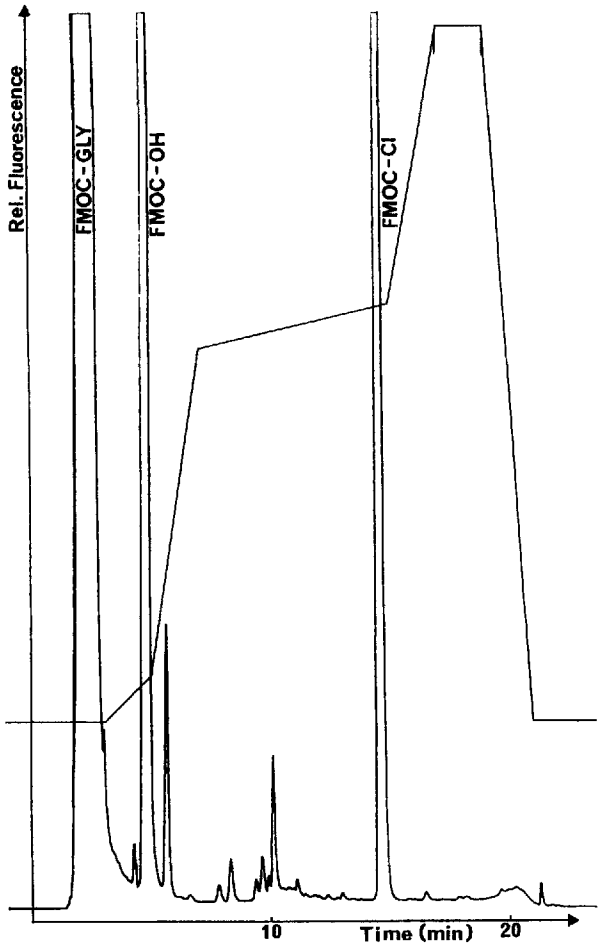

FIGURE 4. Chromatogram of a blank. Glycine is added to derivatize excess FMOC. Conditions see Fig. 3.

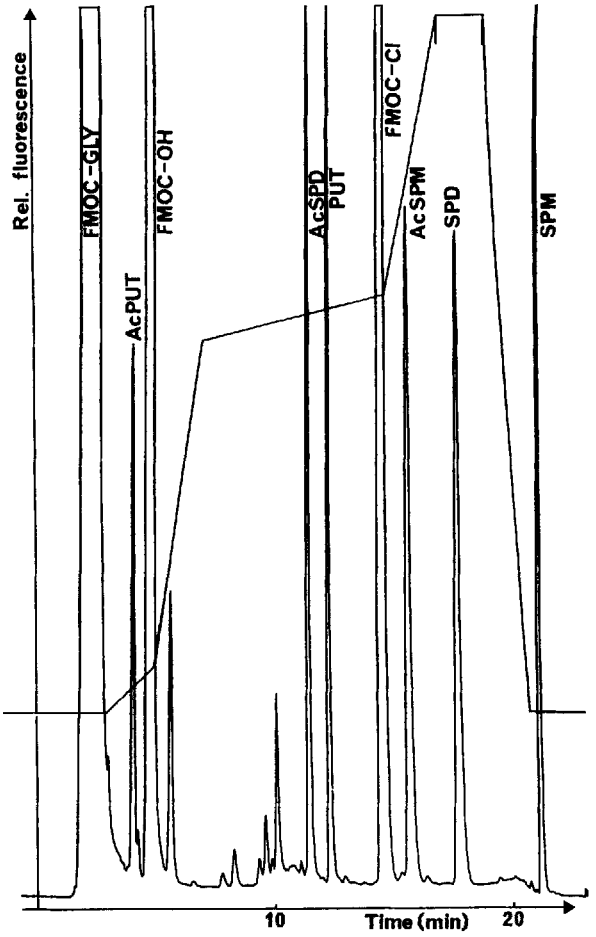

FIGURE 5. Separation of a polyamine standard of 10 pmol per polyamine. Conditions see Fig. 3.

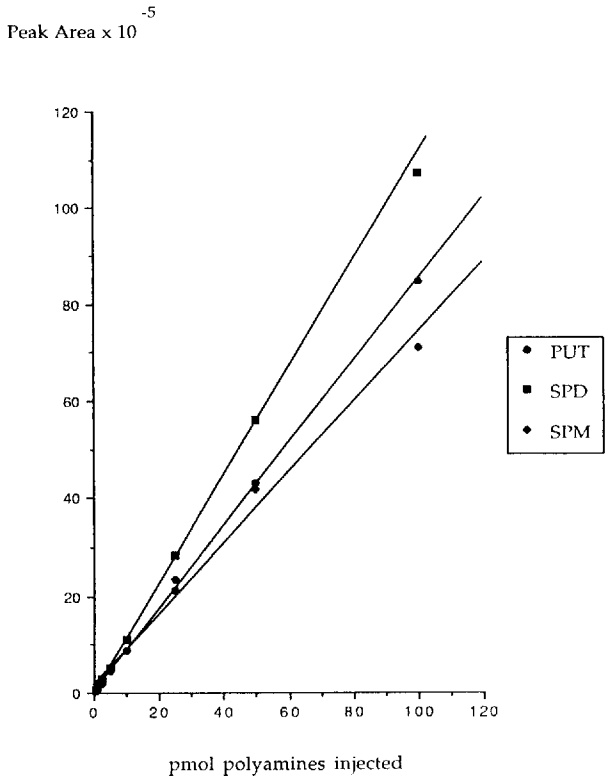

FIGURE 6a . Linearity for putrescine (PUT), spermidine (SPD) and spermine (SPM). 0,5-100 pmol of each polyamine injected.

Stability

Stability of the FMOC derivatives was measured at room temperature (+21° C) every sixth hour for 48 hours. All derivatives were stable for 48 hours except FMOC-SPM which was stable for 18 hours.
Stability was also measured at 0, 24 and 48 hours on a sample kept at +6°C. All polyamine derivatives were stable for at least 48 hours. The results agree well with the stability for FMOC amino acid complexes earlier described (22, 27).

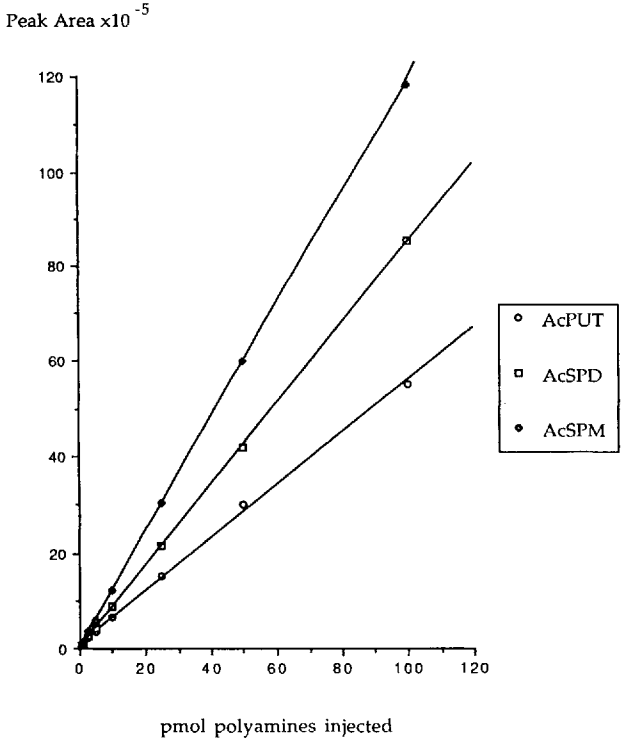

FIGURE 6b. Linearity for N- acetylputrescine (AcPUT), N^8-acetylspermidine (AcSPD) and N^1-acetylspermine (AcSPM). 0,5-100 pmol of each polyamine injected.

TABLE 1

Relative Standard Deviation (RSD%) for Peak Area and Retention Times (R.T.). 10 pmol/polyamine (n=10)

Polyamine	Average Peak Area	RSD%	Average R.T.	RSD% R.T.
AcPUT	663059	4,1	3,02	0,47
AcSPD	878633	3,2	10,52	0,09
PUT	894616	2,4	11,57	0,16
AcSPM	1235716	2,6	14,92	0,32
SPD	1123035	3,8	17,54	0,38
SPM	882303	5,0	20,51	0,10

When no glycine was added to the derivatized sample, the reaction by-products were unstable for more than 2 hours and interfered with the chromatographic separation (Fig. 3).

Separation

Data in Table 1 gives the RSD% of the retention times. The separation reproducibility is excellent with a RSD% of less than 0,5. Figure 5 shows a 21 minute separation of a standard mixture of 10 pmol per polyamine injected.

The Fluorescence Response at Different Mobile Phase Compositions

In the chromatograms here shown, the mobile phase composition rapidly changes over the last eluted compound. The quantum yield of fluorescence is in most cases dependant on the composition of the mobile phase. This was studied in batch measurements where samples of PUT, SPD and SPM. were derivatized and pumped through the fluorescence monitor. The mobile phase consisted of 0, 20, 40, 60, 80 or 100% acetonitrile respectively. The fluorescence response reaches a maximun between 25-50 % acetonitrile (Fig. 7). As followed, the importance of stable retention times is shown.

Sensitivity

The possibility of derivatizing all amino groups with FMOC will theoretically give an enhanced sensitivity compared to OPA, since FMOC can derivatize not only the primary, but also the secondary amino groups (21) in the polyamine molecules. This will give an increase in fluorescence response. Here, the detection limit for AcPUT, AcSPD, PUT and AcSPM is 10 fmol and for SPD and SPM less than 100 amol. However, in routine analysis, it is hard to get reliable results at

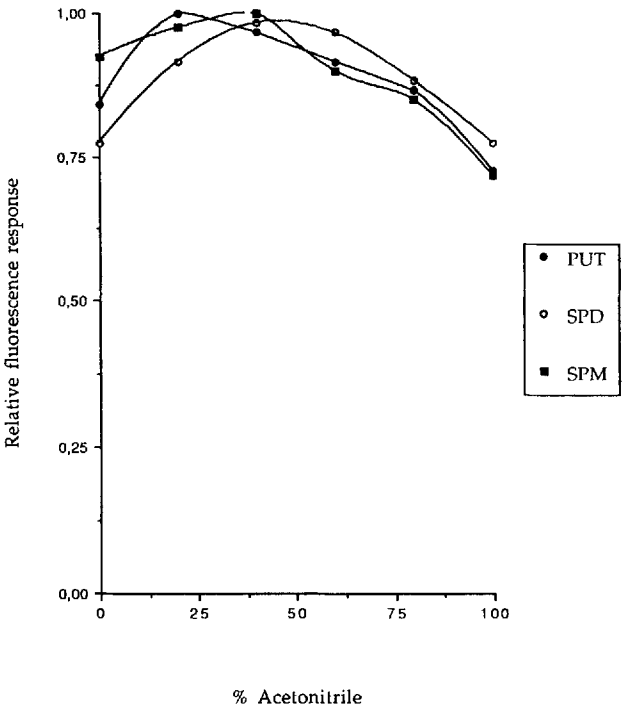

FIGURE 7. The quantum yield of fluorescence response at different mobile phase compositions. At 100 % acetonitrile a decrease in fluorescence response is shown.

these low levels because of interference from background contaminants.

Polyamines in Human Synovial Fluid and Rabbit Lenses

Flescher et al (9) have suggested that polyamines present in synovial fluid from patients with rheumatiod arthritis downregulate interleukin 2 production. Synovial fluid from such patients is a complex biological mixture and it was tested for polyamines.

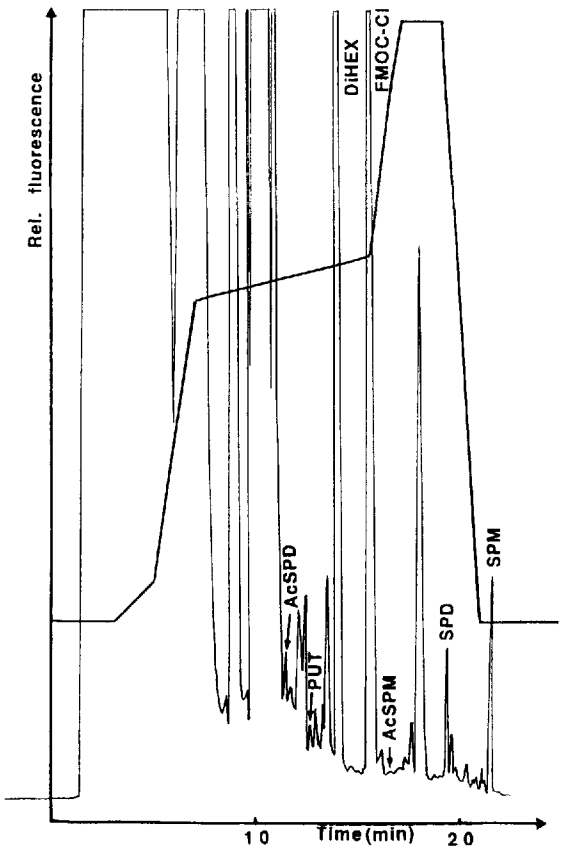

FIGURE 8a. Chromatogram of a deproteinized sample of synovial fluid from rheumatoid arthritis patient (Patient no 3, see Tab. 2) Fluorescence detector; 1/32, 2 mV. Conditions see Fig. 3.

The internal standard DiHex was added to synovial samples from five rheumatiod arthritis patients. Linear (r> 0,998) calibration curves between 25-125 nM were established. After deproteinizing the sample an aliquot of the supernatant was derivatized and directly injected on the column (Fig. 8a). For positive identification, the rest of the sample from patient No. 3 was spiked with 0,25 pmol of each standard

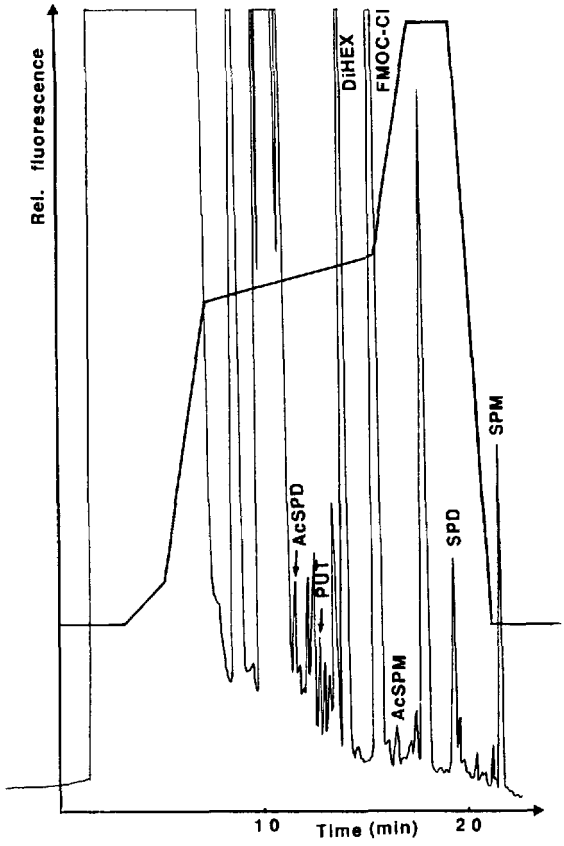

FIGURE 8b. Chromatogram of a deproteinized sample of synovial fluid rheumatoid arthritis. (Patient no 3, see Tab. 2) The sample is spiked with 0,25 pmol of each standard polyamine.
Fluorescence detector; 1/32, 2 mV. Conditions see Fig. 3.

polyamine and injected (Fig. 8b) Here it is not possible to detect AcPUT because of disturbance from other molecules in the sample. Table II shows the polyamines in nmol/ml, determined from 3 repetitive analysis of each sample. Unless otherwise specified, RSD is less than 5%. AcSPM is always the minor polyamine. It is often possible to detect, but the injected amount is below an accurate quantitation. The

TABLE 2

Polyamines in Synovial Fluid of Rheumatoid Arthritis Patients (nmol/ml) and in Rabbit Eye Lens (nmol/g wet weight). (n=3)

	AcSPD	PUT	AcSPM	SPD	SPM
Patient					
No. 1	0,12	nq[1]	nq[1]	0,12	0,18
No. 2	nq[1]	0,15	nq[1]	nq[1]	0,26
No. 3	0,38	0,09	nq[1]	0,59	0,57
No. 3[2]	0,57	0,30	0,21	0,82	0,77
No. 4	0,96	0,68	nq[1]	0,11	0,18
No. 5	nq[1]	0,17	nd[3]	nd[3]	nq[1]
Rabbit lens	0,14	3,72	0,08[4]	3,80	3,96

[1]nq, not possible to quantify with enough accurracy. The sample contains less than 100 fmol injected.

[2]The sample is spiked with 208 pmol of each standard polyamine/ml which gives 0,25 pmol injected (Fig. 8b)

[3]nd, not possible to detect.

[4]RSD is 8,4%

background contaminants interfere and the standard deviation reaches unacceptable values. The polyamine content in synovial fluid varies from patient to patient. It would be interesting to compare these data with the polyamine levels in normal synovial fluid.

The soluble part of rabbit lenses were stirred in Milli Q until dissolved, treated as the synovial fluid and injected (Fig. 9). As shown in Table II, PUT, SPD, and SPM are the three major polyamines and of similar size. The amount of the acetylated polyamines were low, especially AcSPM. AcPUT is not possible to detect. These results are to be compared with the polyamine content in human and bovine lenses (28) where the concentration of SPD and SPM is higher, especially in humans. The extraction procedure employed was also different.

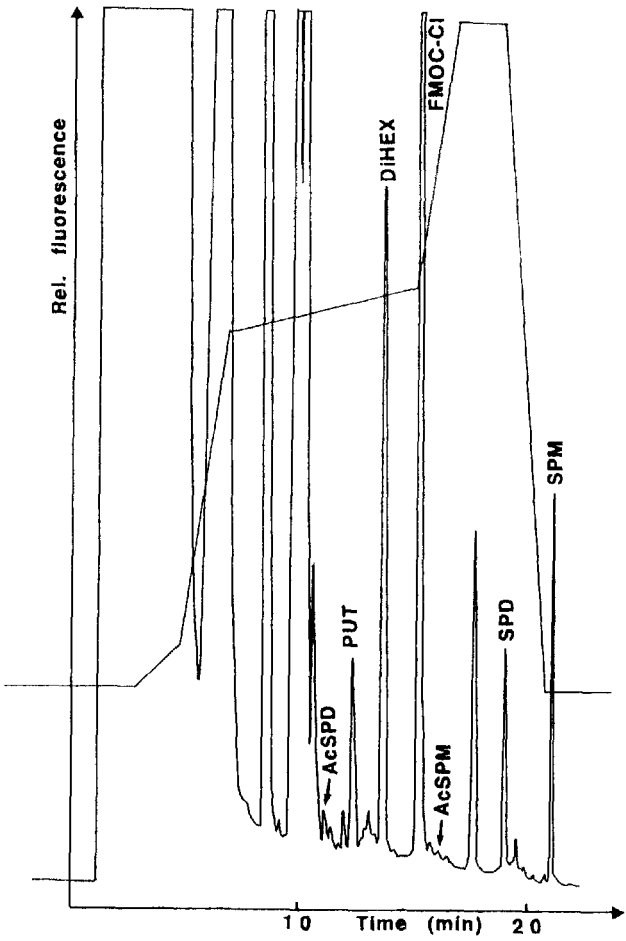

FIGURE 9. Chromatogram of a deproteinized and diluted sample of rabbit eye lens extract. For quantitation see Tab. 2. Conditions see Fig. 3.

CONCLUSIONS

The chromatographic method described in this report is accurate, sensitive and easy to perform. The excess FMOC removal with glycine facilitate the polyamine determination, increases the derivative stability and simplifies the sample handling compared to previously published methods. The method is well suitable for analysis of extra and intracellular AcSPD, PUT, AcSPM, SPD and SPM with a minimum of sample elaboration. For detection and quantitation of AcPUT mobile phase composition must be changed or some kind of selectivity step must be introduced.
The advantages as well as the limits of the method has been evaluated and the method should be useful not only in laboratory research but also in clinical medicine.

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