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REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE SIMULTANEOUS ANALYSIS OF S-ADENOSYLMETHIONINE, ITS METABOLITES AND THE NATURAL POLYAMINES*

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SUMMARY

A method using reversed-phase ion-pair liquid chromatography with dual detection was developed for the simultaneous determination of the S-adenosylmethionine (SAM) analogues and the natural polyamines. The separation is obtained with a gradient elution and by adjusting the concentration of octanesulfonic acid used as ion-pairing agent, the ionic strength of the eluent, the pH and the acetonitrile content of the eluents. The SAM analogues are analyzed by UV detection at 254 nm and the polyamines by fluorescence detection after post-column derivatization with *o*-phthalaldehyde. The method allows the determination of the SAM analogues and the polyamines in one single run by direct injection of tissue extracts. The procedure is applied to the study in rats and in hepatoma tissue culture cells of the biochemical effects of α-difluoromethylornithine, a potent enzyme-activated irreversible inhibitor of ornithine decarboxylase.

INTRODUCTION

S-Adenosyl-L-methionine (SAM) and its metabolites S-adenosyl-L-homocysteine (SAH), decarboxylated S-adenosylmethionine (dc-SAM) and 5'-methylthioadenosine (MTA) are recognized as key intermediates in several biochemical processes [1,2]. SAH is mainly formed in transmethylation reactions which use SAM as a methyl donor [1], dc-SAM acts as the donor of the aminopropyl group for spermidine and spermine biosynthesis [2], and MTA may be formed by various metabolic pathways [3—5]. Fig. 1 summarizes the different biological processes involving SAM and the polyamines.

Several methods for the analysis of SAM and some of its metabolic products

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*Part of these investigations have been presented at the 5th International Symposium on Column Liquid Chromatography, Avignon, France, May 11—15, 1981.

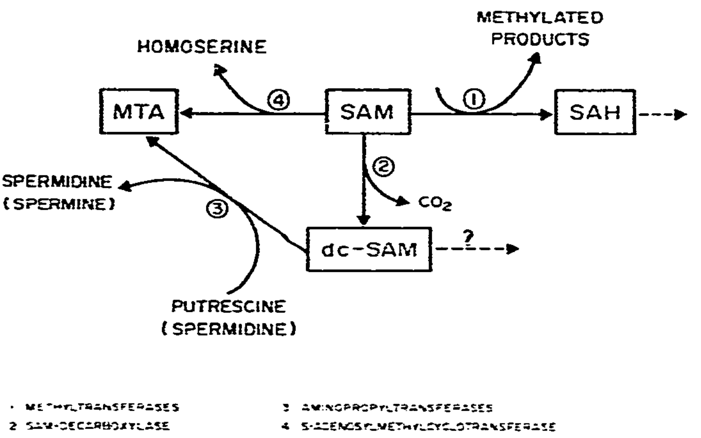
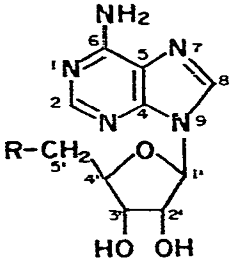


Fig. 1. The major metabolic pathways of S-adenosylmethionine in mammalian tissues.

have been described. Among these, the most recent ones are based on high-performance liquid chromatographic (HPLC) procedures using either cation-exchange [6,7] or reversed-phase [8,9] chromatography with UV detection at 254 nm or fluorescence detection after derivatization before separation [10]. Other column [11,12] and thin-layer [13] chromatographic or electrophoretic procedures have been reported [7,14]. These methods, although quite satisfactory for the analysis of SAM, either need a complex and tedious sample preparation or do not allow the simultaneous determination of all the important SAM metabolites.

Reversed-phase ion-pair liquid chromatography [15,16], which combines the advantages of reversed-phase and ion-exchange chromatography, has proved to be the method of choice for the simultaneous analysis of compounds with various ionizable functions [17—19]. By using a C_{18} column, octanesulfonic



R	ABBREVIATIONS
$\overset{(+)}{\underset{CH_3}{S}}-CH_2CH_2CH(NH_2)COOH$	SAM
$S-CH_2CH_2CH(NH_2)COOH$	SAH
$S-CH_2CH(NH_2)COOH$	SACys
$\overset{(+)}{\underset{CH_3}{S}}-CH_2CH_2CH_2NH_2$	dc-SAM
$S-CH_2CH_2CH_2NH_2$	dc-SAH
$S-CH_2CH_2CH_2NHAc$	N-Ac dc-SAH
$S-CH_3$	MTA
$S-CH_2CH_2CH_2CH_3$	SBA
$S-CH_2CH(CH_3)_2$	SIBA

Fig. 2. Structures and abbreviations of the derivatives and analogues of S-adenosylmethionine.

acid (OSA) as ion-pairing agent and by adjusting the ionic strength, pH and temperature of the eluent, we have achieved separation of the major SAM metabolites. Furthermore, the post-column derivatization procedure with the *o*-phthaldehyde—2-mercaptoethanol reagent and subsequent fluorescence detection is now a well-established procedure for the analysis of amino acids and amines either by ion-exchange [20] or reversed-phase [18,21] chromatography.

The purpose of this work was to establish optimal chromatographic conditions for the determination in a single chromatographic run of the SAM analogues (Fig. 2) by UV detection at 254 nm, and of the polyamines by fluorescence detection after post-column derivatization. The chromatographic method has been applied to studying the effects in vivo of D,L-α-difluoromethylornithine (DFMO), a potent enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC; EC 4.1.1.17) [22]. The SAM, SAH and especially the dc-SAM levels were determined along with the polyamines in various tissues of rats and in cell cultures.

MATERIALS AND METHODS

Chemicals

S-Adenosyl-L-ethionine (SAEt), S-adenosyl-L-homocysteine (SAH), S-adenosyl-L-methionine (SAM) chloride salt, 5′-deoxy-5′-methylthioadenosine (MTA), adenosine, adenine hydrochloride, L-tyrosine (Tyr), L-tryptophan (Trp), L-methionine (Met), putrescine dihydrochloride (Put), spermidine trihydrochloride (Spd), spermine tetrahydrochloride (Spm) and 2-mercaptoethanol were products of Sigma (St. Louis, MO, U.S.A.). S-Adenosyl-D-cysteine (SACys), S-butyladenosine (SBA) and S-isobutyladenosine (SIBA) were supplied by Prof. E. Lederer (Gif-sur-Yvette, France), and the N-acetylputrescine (N-Ac-Put), N^1- and N^8-acetylspermidine (N^1-Ac- and N^8-Ac-Spd) were provided by Dr. N. Seiler (MIRC, Strasbourg, France). S-5′-Adenosyl-(5′)-3-methylthiopropylamine bisulfate (dc-SAM), S-5′-adenosyl-3-thiopropylamine bisulfate (dc-SAH) and S-5′-adenosyl-3-N-acetylthiopropylamine (N-Ac dc-SAH) were prepared following published procedures [23,24]. D,L-α-Difluoromethylornithine (DFMO, RMI 71782) was synthesized in our Centre [25]. Octanesulfonic acid (OSA) sodium salt was obtained from Eastman Kodak (Rochester, NY, U.S.A.); *o*-phthalaldehyde (OPA) and ethylenediaminetetraacetic acid (EDTA) disodium salt were from C. Roth (Karlsruhe, G.F.R.). Sodium dihydrogen phosphate (NaH_2PO_4), acetonitrile, boric acid, the wetting agent Brij-35, and all the other chemicals were from E. Merck (Darmstadt, G.F.R.).

Animals

Male Sprague—Dawley rats (80—300 g) from Charles River (Saint Aubin-les-Elbeuf, France) were used throughout these studies.

Cell cultures

Morris hepatoma 7288 C tissue culture (HTC) cells were used [34].

Chromatographic system

The high-performance liquid chromatograph consisted of two Model 6000A pumps, a Model 660 solvent programmer, a Model 440 UV-absorbance detector operating at 254 and 280 nm, an automatic sample processor WISP and a μBondapak C_{18} column (10 μm particle size, 30 cm × 3.9 mm I.D.), all from Waters Assoc. (Milford, MA, U.S.A.). A guard column (7 cm × 2 mm I.D.) filled with Co:Pell ODS from Whatman (Clifton, NJ, U.S.A.) was used throughout these studies to protect the main column. The column was always thermostated in a jacket with a circulating water bath. The fluorescent derivatives were obtained by continuously mixing through a T-piece the effluent of the UV cells with the OPA reagent. A piston pump (Dosapro; Milton Roy, Riviera Beach, FL, U.S.A.) was used for pumping the OPA reagent and a pulse dampener was inserted before the mixing T-piece. The fluorescence detector was an Aminco Fluoromonitor (American Instruments Co., Silver Spring, MD, U.S.A.) fitted with a 70-μl flow cell. A Corning 7.51 filter was placed in the excitatory beam and a Wratten 2A filter was inserted in the emitted light. The optimal wavelengths for the OPA derivatives are 340—345 nm and 455 nm for the excitation and emission, respectively. The signal of the UV detector (254 nm) was recorded and integrated with an SP4100 digital integrator from Spectra Physics (Santa Clara, CA, U.S.A.). The signals of the UV detector at 280 nm and of the fluorescence detector were recorded on an Omniscribe recorder from Houston Instruments (Austin, TX, U.S.A.). The peak areas of the fluorescence detection were determined with an Autolab System I integrator from Spectra Physics.

Mobile phases

A gradient elution system was used consisting of two mobile phases. Mobile phase A was obtained by the addition of 20 ml of acetonitrile to 980 ml of 0.1 *M* NaH_2PO_4 and contained $8 \cdot 10^{-3}$ *M* OSA and $1 \cdot 10^{-4}$ *M* EDTA. The pH was adjusted to 2.55 with 3 *M* H_3PO_4. Mobile phase B was a 70:30 (v/v) mixture of 0.2 *M* NaH_2PO_4 and acetonitrile with $8 \cdot 10^{-3}$ *M* OSA and the pH was adjusted to 3.10 with 3 *M* H_3PO_4. A linear gradient was used starting with initial conditions consisting of 85% of eluent A and 15% of eluent B and leading in 30 min to the final conditions consisting of 15% of A and 85% of B. At this time, automatic resetting, through the WISP, to the initial conditions occurred, followed by a waiting time of 15 min before starting the next injection. The use of the automatic sample injector to control the solvent gradient (start, resetting and waiting time) allowed us to obtain excellent reproducibility of the chromatographic conditions.

The acetonitrile was distilled over phosphorus pentoxide and the water was distilled over phosphoric acid before use.

The various eluents used with different concentrations of OSA, various ionic strengths of the buffer and pHs were prepared in a similar fashion.

o-Phthalaldehyde reagent

The reagent was prepared according to a published procedure [20] with slight modifications. A 0.5 *M* solution of boric acid was adjusted to pH 10.4

with a 45 g/l KOH solution. After filtration through a Millipore 0.45-μm HA filter, 2.5 ml of 2-mercaptoethanol, 3 ml of Brij and 800 mg of *o*-phthalaldehyde dissolved in 10 ml of ethanol were added. The final solution was protected from light.

Calibration standards

Stock solutions of the different SAM analogues and of the polyamine derivatives were prepared by dissolving 0.5—3 mg of the different compounds in 25 ml of 0.01 *M* $HClO_4$ with 0.05% (w/v) $Na_2S_2O_5$ and 0.01% (w/v) EDTA. From these stock solutions adequate standard solutions were obtained by dilution. Fresh stock solutions were prepared every 2—3 weeks and stored at 0—5°C. Aliquots of 5—100 μl of these standard solutions were injected on to the column.

Sample preparation

The rat tissues were homogenized in 5 ml of 0.2 *M* $HClO_4$ or 0.3 *M* trichloroacetic acid. After centrifugation at 3000 *g*, the supernatants were filtered through a Millipore membrane (0.22 μm). HTC cells were collected by centrifugation, washed twice with phosphate buffer and disrupted by sonication in 0.05 *M* sulfuric acid. The proteins were precipitated by adding 0.2 *M* $HClO_4$ and were discarded by centrifugation; 10—100 μl of these acid extracts were applied to the column.

Recovery experiments

Known amounts of the SAM analogues were dissolved in the 0.2 *M* $HClO_4$ solution used for the tissue homogenization. The samples were treated as described above. The recoveries were determined by subtracting the values obtained for control tissues of rats of similar size. The recovery experiment was performed only with prostates.

Calculations

The response of the UV detector at 254 nm was linear from 50 to 2000 pmoles of the various analogues by measuring the peak heights or the peak areas. For the polyamine analogues, the response of the detector was linear from 200 to 5000 pmoles by measurement of the peak areas. However, for Spd and Spm especially, the peak height response, due to the tailing of the peaks, was not linear. Therefore all the values presented for the polyamines were obtained by peak area measurements. An external standard containing all the compounds of interest was injected every eighth sample in order to correct for the slight variations in both sensitivity, mainly for the fluorescence detection, and in retention times. As the recovery of the different compounds was found to be quantitative ($\geqslant$ 95%), no internal standard was added and no correction for recovery was performed. The commercially available SAM contained 30% of impurities, mainly MTA but together with several other unknown compounds, as shown by HPLC. It was therefore necessary to correct for these impurities when calculating the results. The actual amounts of the different compounds were expressed in nmole/g of wet tissue.

RESULTS AND DISCUSSION

Determination of the chromatographic conditions

The goal of this study was to achieve separation of the major SAM metabolites and of the most important polyamines in one single chromatographic run. Analysis of some SAM analogues, as outlined above, has already been achieved by cation-exchange or reversed-phase HPLC [6–9]. Moreover, numerous reports using mainly cation-exchange [20,26] chromatography have described the analysis of amino acids and polyamines. Recently, reversed-phase ion-pair chromatography has been used for the analysis of polyamines [21].

In order to obtain the desired separation of all the compounds of interest, we have used C_{18} reversed-phase ion-pair chromatography and systemically studied the different factors which control the retention of these different compounds. The great variations in lipophilicity between the various compounds, i.e. MTA and SIBA, and the pronounced differences in their ionization properties, i.e. SAH and spermine, precluded the use of an isocratic elution system. The use of acetonitrile as organic solvent proved the most satisfactory; nevertheless, it was found necessary to add EDTA to eluent A in order to compensate for the baseline drift observed at 254 nm. The use of NaH_2PO_4 as buffer is advantageous due to its low absorbance at 254 nm and especially to the ease with which the pH can be varied between 4 and 2.4 by addition of phosphoric acid. The effect of the chain length of the reversed-phase has not been studied, but the results obtained with μBondapak C_{18} in respect to peak tailing [27] proved superior to some other C_{18} phases with higher carbon loading. Octanesulfonic acid, which we have used extensively in our previous studies [17,28], was chosen as the ion-pairing agent.

Effect of concentration of octanesulfonic acid

Fig. 3 shows that the retention times of the different compounds strongly depend on the amount of OSA in the concentration range studied of 4–8 m*M*. All the chromatograms were obtained with the same gradient pattern as described in the experimental procedure but the eluents were slightly different; the ionic strengths of eluents A and B were 0.1 *M*, the pH was 2.80 for A and 3.00 for B and the temperature was 30°C. As expected, the retention times of the amines increased more rapidly than those of the amino acids. The best separation pattern was obtained at 8 m*M* OSA and this concentration was used for all the remaining studies.

Effect of pH

As expected, the retention behaviour of the different compounds was strongly affected by the pH of eluents A and B. Fig. 4 illustrates the variation of the capacity factor k' for several SAM analogues and tryptophan. For these studies, the ionic strength of eluents A and B was 0.1 *M*, the temperature was 28°C and the OSA concentration was 8 m*M*. The pH of both eluents A and B was adjusted to the values shown in Fig. 4 with concentrated or diluted phosphoric acid. The retention times of the compounds containing a carboxylic acid group, i.e. SAM, SAH and Trp, strongly depend on the pH of the eluents; the retention times of the other SAM derivatives, i.e. dc-SAM, dc-SAH, MTA

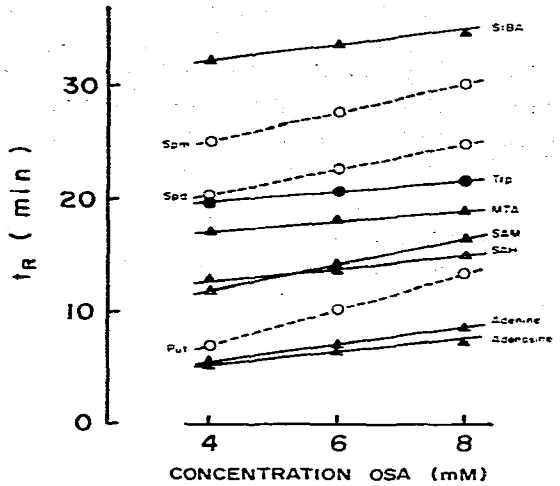


Fig. 3. Relationship between the retention times (t_R) and the concentration of OSA of the mobile phases. Chromatographic conditions: column, gradient, flow-rate and acetonitrile as described in the Methods section; ionic strength of eluents A and B = 0.1 *M*; pH (A) = 2.80, pH (B) = 3.00; temperature = 30°C.

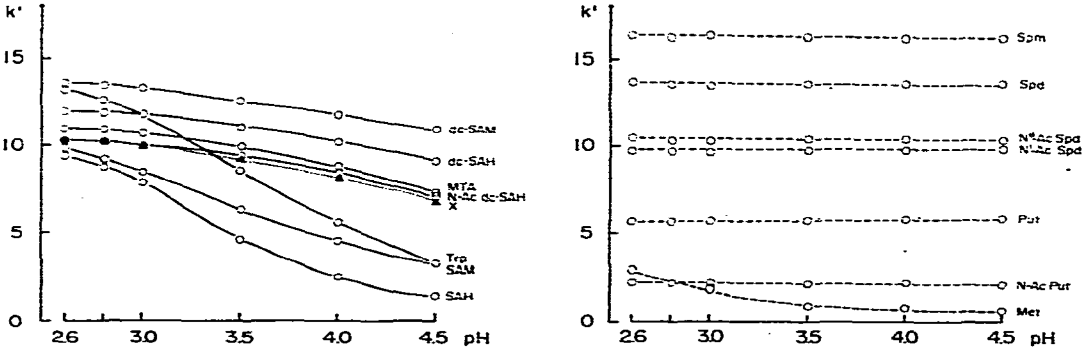


Fig. 4. Relationship between the capacity factor *k'* and the pH of the mobile phases. Chromatographic conditions were as described in the Methods section, except ionic strength of the eluents A and B = 0.1 *M*, temperature = 28°C, and the pH was adjusted to the different values with phosphoric acid.

and N-Ac dc-SAH, only moderately increase with decreasing pH. This is probably due to the protonation of the amino group of the adenine ring. Moreover, the polyamines that contain amino groups but no acid functions are completely protonated in the pH range studied and show no variation in retention times with pH changes. It is obvious from Fig. 4 that, at pH ⩾ 4.0, the retention times of SAM, SAH and Trp are too short to allow their clear separation from all the poorly retained amino acids that occur in biological samples. Therefore we have chosen a fairly low pH for eluents A (2.55) and B (3.10), which give optimal separation of all the SAM analogues. Compound X corresponds to an as yet unidentified metabolite which appeared in biological samples after treatment with an ODC inhibitor.

Ionic strength of the buffer

Several hypotheses have been proposed to explain the reversed-phase ion-pair mechanism [29,30]. It is now recognized that a dual mechanism of simple ion-pairing and dynamic ion exchange does not fully account for the experimental results [31,32]. Nevertheless, Fig. 5a and b show that at given pHs (2.55 for A and 3.10 for B), temperature 28°C, concentration of OSA ($8 \cdot 10^{-3}$ *M*) and flow-rate (1.5 ml/min), the capacity factor k' strongly decreases with increasing buffer strength. This decrease depends essentially on the number of positive charges contained in a given compound. The effect is the most pronounced for the polyamines (Spm, Spd, Put) which are tetra-, tri-, or diprotonated, respectively, at the pH considered and for the sulfonium derivatives SAM and dc-SAM. It is of interest to note the difference in variation of retention times between dc-SAM and dc-SAH or SAM and SAH, which is due solely to the presence of the positively charged sulfonium group. The retention times of the simple amino acids tryptophan, tyrosine and methionine, and of the adenosine derivatives MTA, SIBA and SBA, are less affected by changes in ionic strength. The observed decrease in retention time with increasing concentration of Na^+ is in good agreement with a dynamic ion-exchange mechanism.

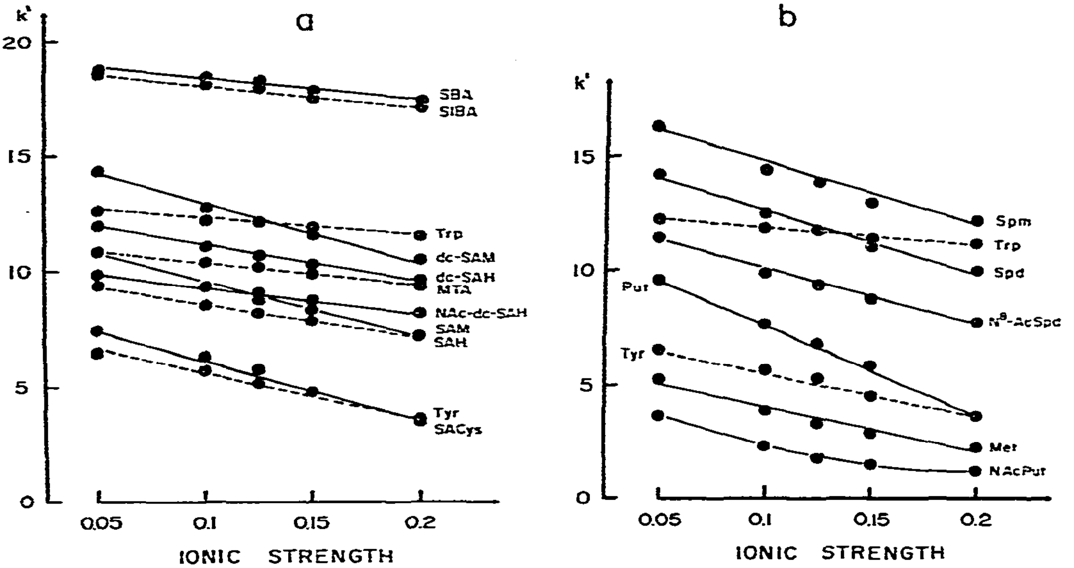


Fig. 5. Dependence of the capacity factor k' on the ionic strength of the eluents. Panel a represents the compounds studied at 254 nm and panel b those observed by fluorescence detection. Chromatographic conditions were as described in the Methods section except for the ionic strengths of the different buffers which were adjusted to the given values.

In view of these results, a 0.1 *M* buffer was selected for eluent A whereas for eluent B a final buffer strength of 0.14 *M* was found to be optimal.

Effect of temperature

It is well known that the efficacy of reversed-phase columns increases with temperature. Furthermore, the retention behaviour of different solutes in ion-exchange chromatography strongly depends on the temperature. Fig. 6a and b present the variation of k' with temperature between 20 and 50°C for the SAM analogues and the polyamines, respectively. For these studies, the optimal chromatographic conditions previously defined were used. The buffer strength of A was 0.1 *M* whereas the final buffer strength of B, obtained with a 70:30 (v/v) mixture of 0.2 *M* buffer with acetonitrile, was 0.14 *M*. The pH of eluent A was 2.5 and for B 3.10, and the OSA concentration was $8 \cdot 10^{-3}$ *M*. A pronounced decrease in the capacity factor of the amino acids Tyr and especially Trp as the temperature increases is observed. This is probably due to the decrease of the apparent pK_a of the carboxylic acid group [33]. From these results, it is obvious that temperatures around 20 and 40°C can be used to achieve separation of Trp and Spd. A temperature of 40°C which combined better efficacy and shorter retention times was chosen.

Fig. 7 shows the chromatograms obtained with UV (254 and 280 nm) and fluorescence detection using the chromatographic conditions previously defined. All the SAM analogues and the major polyamines along with Tyr and Trp are clearly separated. The use of multiple detection is a major advantage especially for the simultaneous analysis of compounds with such different properties. The polyamines do not absorb at 254 or 280 nm, whereas the SAM analogues react very weakly if at all with OPA under the conditions used. Tyr and Trp, which show a stronger absorption at 280 nm than at 254 nm, can be analyzed with all three detection modes. Under the same chromatographic conditions, SAEt has a retention time of 12.65 min and is eluted between SAM and N-Ac dc-SAH.

The results described above led us to choose the following chromatographic conditions: linear gradient leading in 30 min from initial conditions with 85%

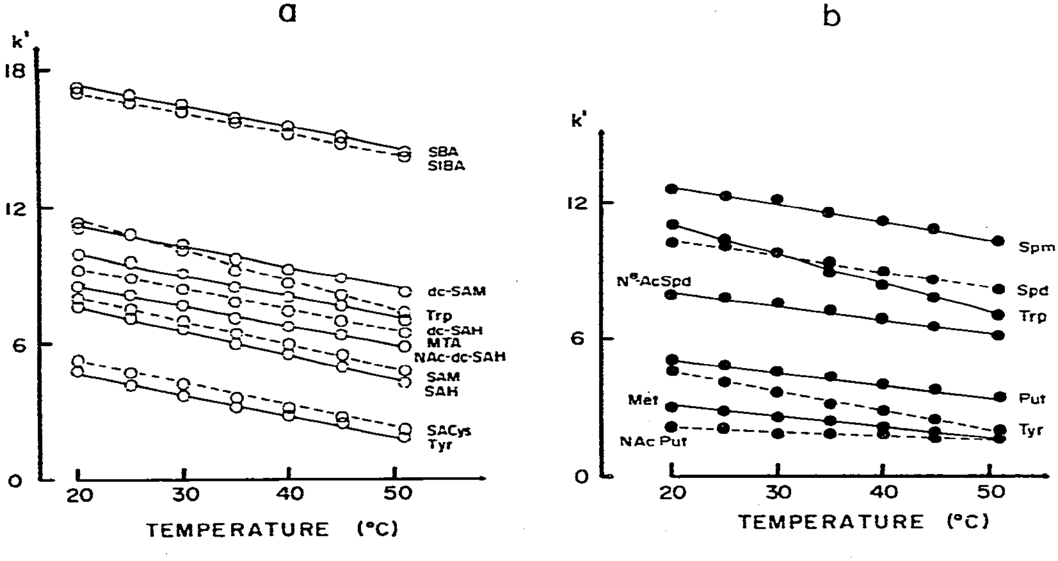
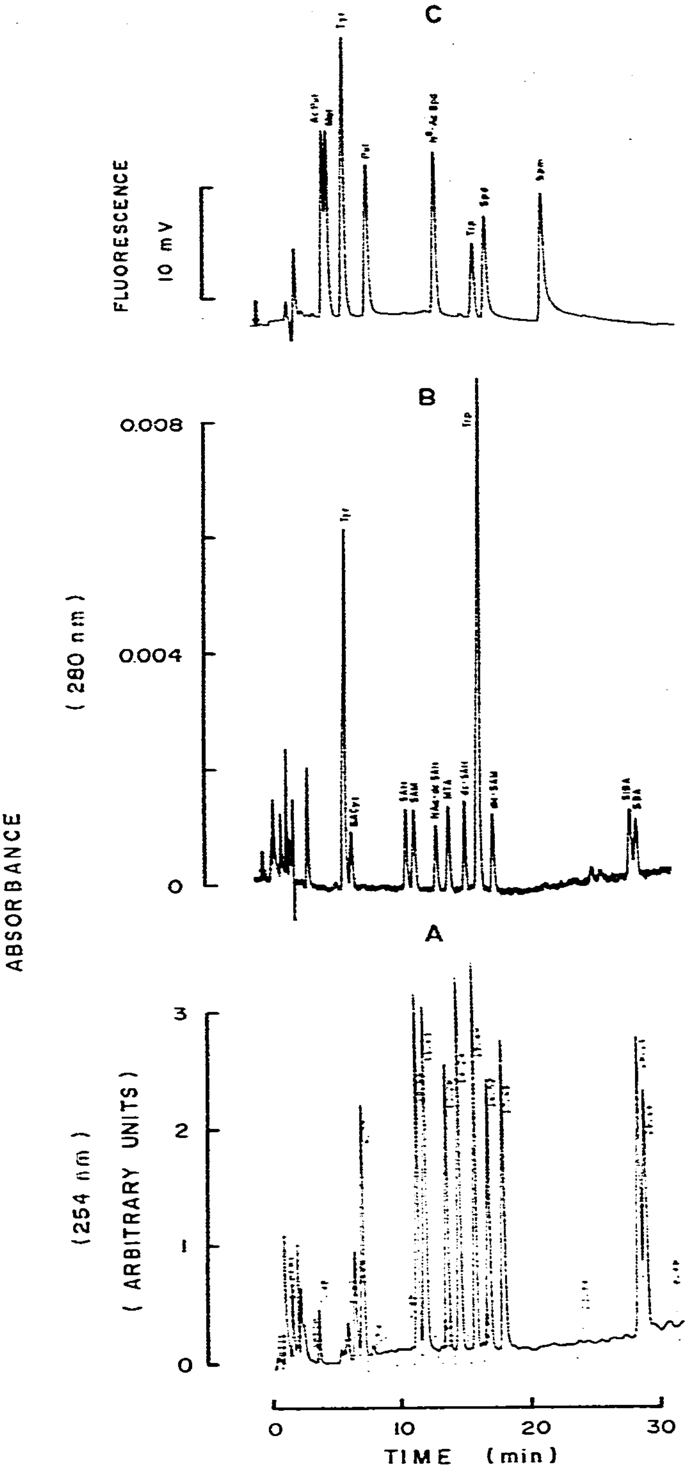


Fig. 6. Relationship between the capacity factor k' and temperature of the column for the compounds observed by UV detection at 254 nm (a) and fluorescence detection (b). Chromatographic conditions were as described in the Methods section.


C
FLUORESCENCE
10 mV
Ac Put
Tyr
Put
N⁸-Ac Spd
Trp
Spd
Spm
B
0.008
0.004
0
ABSORBANCE
(280 nm)
Tyr
SAH
SAM
MTA
Trp
A
3
2
1
0
(254 nm)
(ARBITRARY UNITS)
0
10
20
30
TIME (min)

A and 15% B to final conditions with 15% A and 85% B, at a flow-rate of 1.5 ml/min and a temperature of 40°C. These conditions were used for the analysis of most of the biological samples.

APPLICATIONS

Effect of repeated treatment of rats with DFMO on SAM analogues in prostate

The effect of a three-day treatment with 2% (w/v) DFMO in drinking water on SAM and its analogues in the prostate of rats was monitored. Fig. 8 shows typical chromatograms obtained with UV detection at 254 nm of a treated (A) and control (B) prostate sample. Tyr, Trp, SAH and SAM are clearly detectable in the control sample whereas MTA ($t_R \approx 20$ min) and dc-SAM ($t_R \approx 24.5$ min) are at the limit of detection. After treatment SAH and SAM are not significantly affected whereas two other peaks strongly increase. The peak whose retention time is around 24.5 min most probably represents dc-SAM. Indeed it has the same UV absorbance pattern ($\epsilon_{254} > \epsilon_{280}$) and the same chromatographic behaviour with change of pH of eluents as those of dc-SAM (see Fig. 4). The increase in dc-SAM levels in treated prostates as compared to control prostates can be explained by inhibition of ODC which leads to a diminution of Put and Spd levels [22,34,35], and to an increase in the SAM-decarboxylase activity [36]. The identity of the peak indicated by X whose retention time is very similar to that of N-Ac dc-SAH has not been established. Nevertheless, the UV absorbance ($\epsilon_{254} > \epsilon_{280}$), the failure to react with OPA under the conditions used, and the chromatographic behaviour (see Fig. 4, compound X) are in favor of a derivative of SAM. Further studies need to be undertaken in order to isolate this compound and elucidate its structure. The values for SAM, SAH, dc-SAM, and MTA along with those of Tyr and Trp are presented in Table I.

SAM analogues and polyamines in testis, thymus, pancreas and prostate of rats after chronic treatment with DFMO

The chromatograms of extracts of testis with UV (254 nm) and fluorescence detection from controls and rats treated during 12 days with 2% (w/v) DFMO in the drinking water are shown in Figs. 9 and 10. SAH, SAM and dc-SAM along with Tyr and Trp are clearly observed with UV detection at 254 nm in the control tissue (Fig. 9B). A peak with a retention time (t_R = 15.55 min) very close to that of dc-SAH is present. In testis from DFMO-treated rats, dc-SAM and X increased (Fig. 10B) whereas Put and Spd markedly decreased

Fig. 7. Chromatograms of the standard solution of SAM analogues and polyamines by UV detection at 254 nm (A), 280 nm (B) and fluorescence detection (C) after o-phthalaldehyde derivatization. This standard was used as external standard for the analysis of the different tissues. For details of the chromatographic conditions see the Methods section. The assignment of the peaks is the same at 254 nm (A) and at 280 nm (B) (amounts in nmoles in parentheses): Tyr (2.13); SACys (0.12); SAH (0.14); SAM (0.18); N-Ac dc-SAH (0.16); MTA (0.14); dc-SAH (0.15); Trp (0.78); dc-SAM (0.16); SIBA (0.17); SBA (0.15). Fluorescence detection: Ac-Put (0.63); Met (1.05); Tyr (2.13); Put (0.58); N^8-Ac-Spd (0.45); Trp (0.78); Spd (0.47); Spm (1.16). The sensitivity of the Aminco Fluoromonitor was set at 100 with the recorder scale at 50 mV.

TABLE I

EFFECT OF DFMO (REPEATED TREATMENT)* ON SAM ANALOGUES, Tyr AND Trp IN PROSTATE OF RATS

The values are expressed in nmoles/g wet weight ± S.D. and the organ weight in g ± S.D. (*n* = 5).

	SAM	SAH	dc-SAM	MTA	Tyr	Trp	Organ weight
Controls	49.8 ± 14.3	11.3 ± 6.7	1.65 ± 0.40	0.34 ± 0.13	977 ± 94	249 ± 31	0.57 ± 0.06
DFMO-treated	79.4 ± 17.9	10.4 ± 1.0	888 ± 217	0.40 ± 0.07	397 ± 2.10	94.4 ± 52.7	0.43 ± 0.07

*2% (w/v) in drinking water for three days.

TABLE II

EFFECT OF CHRONIC TREATMENT* WITH DFMO ON SAM ANALOGUES AND POLYAMINES IN VARIOUS TISSUES OF RATS

Results are expressed in nmoles/g ± S.D. and the tissue weights in g ± S.D. (*n* = 5).

Tissue	Treatment	SAM	SAH	dc-SAM	Put**	Spd**	Spm**	Organ weight
Testis	Control	15.99 ± 1.24	3.36 ± 0.53	2.17 ± 0.36	14.7 ± 3.1	261 ± 18	652 ± 38	0.87 ± 0.09
	DFMO	16.58 ± 1.17	3.78 ± 0.68	8.34 ± 2.9	<1	106 ± 24	708 ± 21	0.83 ± 0.06
Thymus	Control	55.7 ± 7.9	2.13 ± 0.50	<0.12 ± 0.05	276 ± 20	2362 ± 337	1110 ± 139	0.63 ± 0.07
	DFMO	56.4 ± 2.2	2.89 ± 0.69	4.57 ± 1.80	51 ± 12	1005 ± 135	1478 ± 70	0.43 ± 0.05
Pancreas	Control	31.2 ± 2.1	6.30 ± 0.66	0.60 ± 0.16	28.5 ± 5.4	4017 ± 743	1026 ± 285	0.83 ± 0.04
	DFMO	35.84 ± 2.8	5.87 ± 0.58	0.84 ± 0.09	16.0 ± 4.0	4544 ± 870	1343 ± 298	0.66 ± 0.11
Prostate	Control	75.9 ± 7.9	4.54 ± 0.98	4.83 ± 0.97	459 ± 111	6241 ± 1536	2942 ± 173	0.17 ± 0.03
	DFMO	84.6 ± 8.5	10.82 ± 2.3	2062 ± 464	143 ± 67	356 ± 148	2328 ± 321	0.09 ± 0.02

*Rats were treated for 12 days with 2% (w/v) DFMO in the drinking water.

**Results of Put, Spd and Spm are obtained from fluorescence detection.

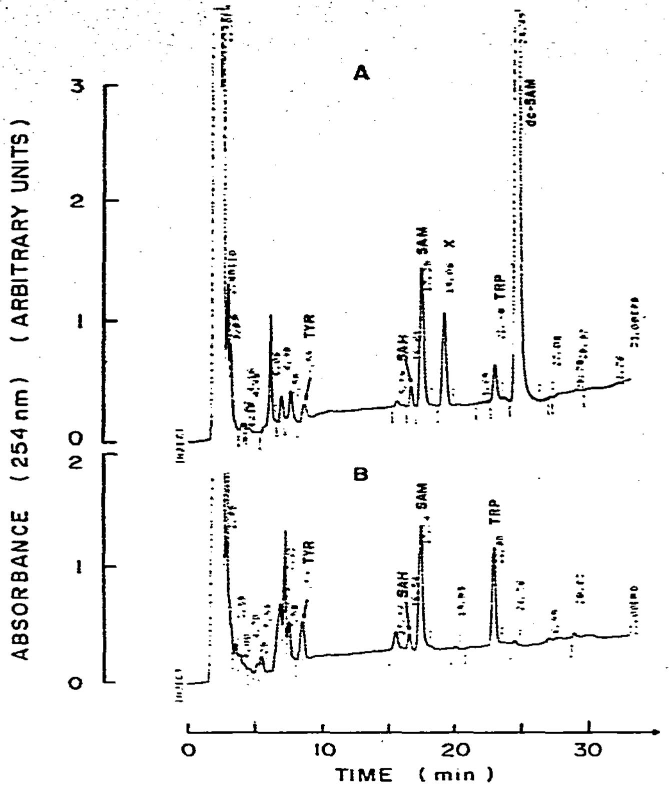


Fig. 8. Chromatograms of SAM derivatives with UV detection at 254 nm from rat prostate after treatment for 3 days with DFMO (A) and control (B). A 25-µl aliquot of the supernatant obtained after homogenization in 0.2 *M* $HClO_4$, centrifugation and filtration, was injected into the column. Chromatographic conditions were as described in the Methods section except for the ionic strength of eluent B, 0.1 *M* instead of 0.14 *M*, and temperature = 28°C.

and Spm remained unchanged (Fig. 10A). Similar changes were observed in the thymus, where dc-SAM was only detected in the tissue of treated rats when Put and Spd decreased and Spm remained unchanged (when expressed in nmole per total organ but not when expressed in nmoles/g of tissue). Analysis of the pancreas of the same rats showed no marked effect of DFMO treatment either on dc-SAM or on Put or Spd levels. The concentrations of the SAM analogues and of the polyamines in the prostates of the same rats showed similar alterations following DFMO treatment; however, the changes in dc-SAM were more pronounced than in the other tissues. The experimental results for all the tissues examined are summarized in Table II.

In general, the results obtained for control rats are in fairly good agreement with published values, only the values for SAH and SAM (3.4 and 16.0 nmoles/g) in testis are slightly lower than those obtained by Eloranta [37] (5.09 and 21.3) using a different method. The values obtained for different control prostates (Table III) show some variation, which seems to depend on the size of the organ and the age of the rats. SAM, SAH and dc-SAM levels in

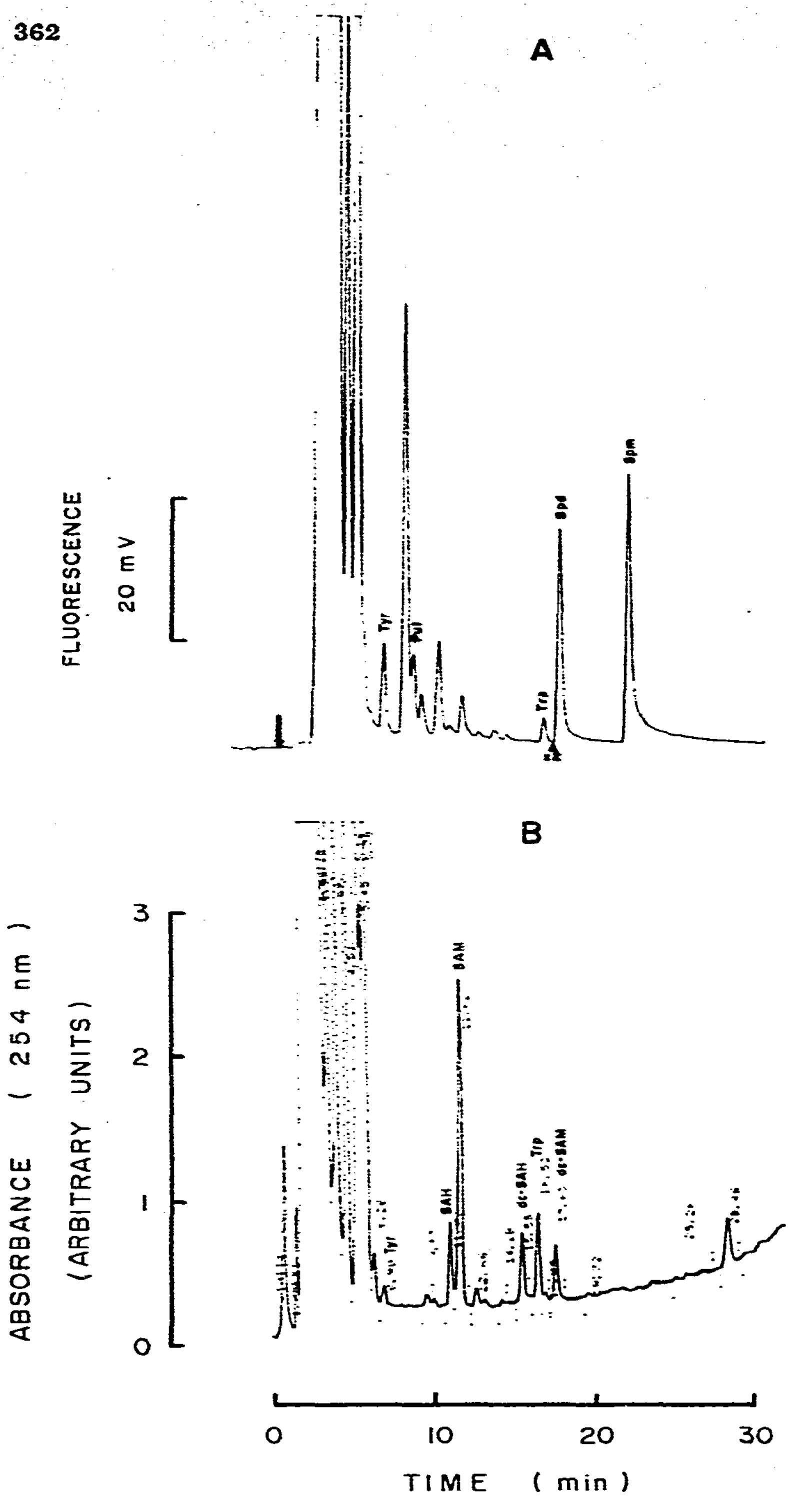


Fig. 9. Chromatograms obtained with UV (254 nm) detection (B) and fluorescence detection (A) of rat testis control. A 50-μl aliquot of the supernatant, obtained as described in the sample preparation section, were injected into the column. Chromatographic conditions were similar to those of Fig. 7.

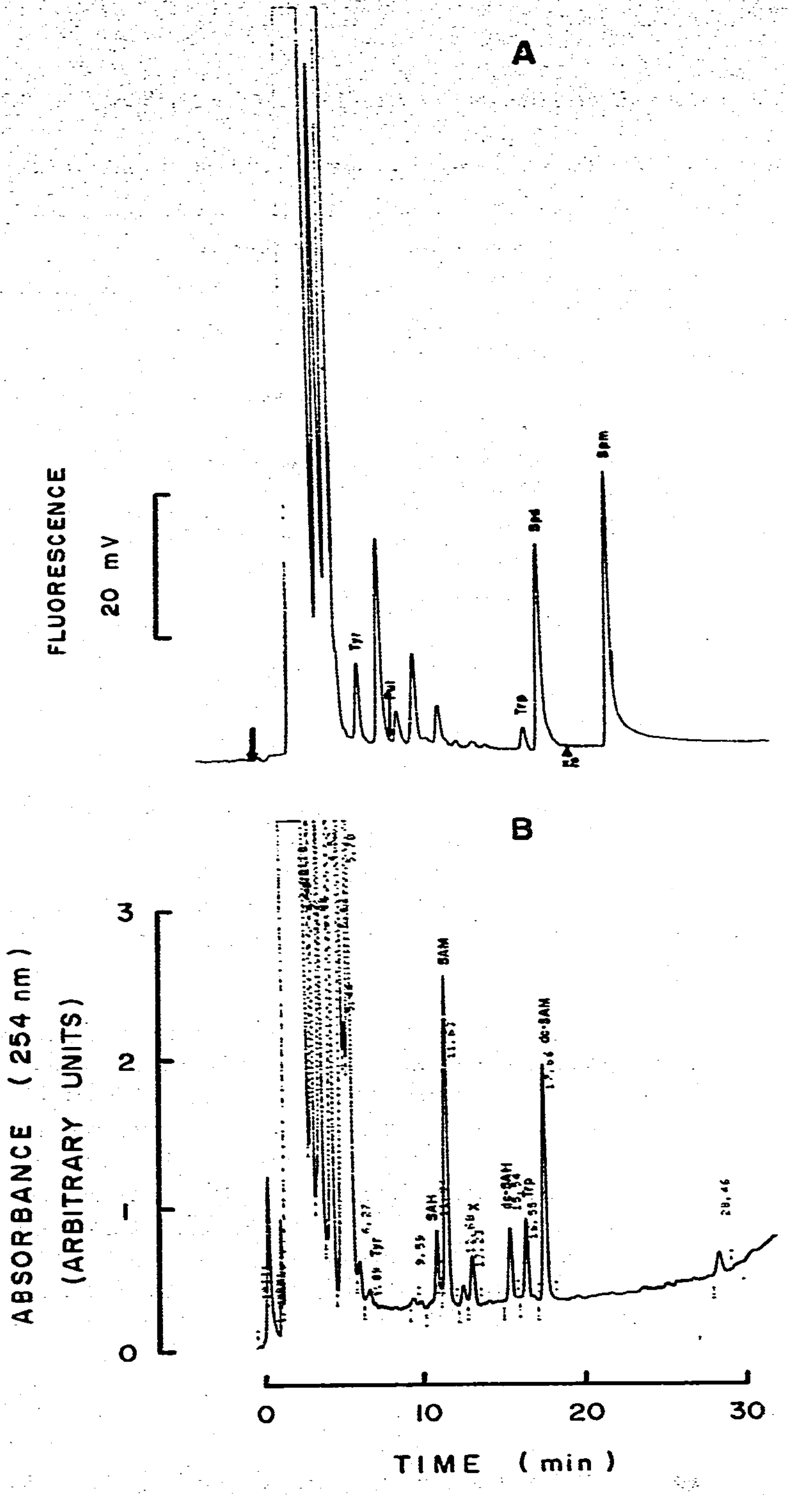


Fig. 10. Chromatograms obtained with UV (254 nm) detection (B) and fluorescence detection (A) of testis of rats treated for 12 days with DFMO (2%, w/v) in the drinking water. For further details see Fig. 9.

TABLE III

CONCENTRATION OF SAM, SAH AND dc-SAM IN PROSTATE OF CONTROL RATS OF DIFFERENT SIZES

Values are expressed in nmoles/g wet weight ± S.D. for five rats in each series unless otherwise indicated.

SAM	SAH	dc-SAM	Organ weight (rat size)*	Reference
49.8 ± 14.3	11.3 ± 6.7	1.65 ± 0.40	0.57 ± 0.06 (329 ± 16)	Present work
76.7 ± 18.6	5.4 ± 0.5	1.49 ± 0.52	0.29 ± 0.06 (237 ± 8)	Present work
75.9 ± 7.9	4.54 ± 0.98	4.83 ± 0.97 ($n = 4$)	0.17 ± 0.03 (188 ± 7)	Present work
60.7 ± 11.6	3.14 ± 0.63	4.13 ± 1.34	0.06 ± 0.01 (81 ± 2)	Present work
59.5	10.4		(6-week-old rats) (ca. 160 g)	37
57.4 ± 4.6**		2.06 ± 0.34	(200—300 g rats)	7

*Rat size expressed by the weight in g ± S.D.
**Mean ± S.D. for at least four measurements from different animals [7].

prostates of rats of 300 g weight were in good agreement with those obtained by Hibasami et al. [7] and Eloranta [37] for rats of similar size.

The values of the polyamines are in good overall agreement with published values obtained by ion-exchange chromatography [22], only our values for Spm in testis and thymus are somewhat higher. The discrepancy may be explained by differences in organ size.

SAM analogues and polyamines in HTC cells after treatment with DFMO

Figs. 11 and 12 show chromatograms of extracts from control and DFMO-treated HTC cells, respectively, with UV detection at 254 nm (A), 280 nm (B) and fluorescence detection (C). SAH, SAM, Tyr and Trp are clearly detected by UV absorbance and Put, Spd and Spm are demonstrated by fluorescence detection. After treatment with DFMO, the chromatographic pattern was modified. As was observed previously in several organs of rats after treatment with DFMO, dc-SAM strongly increased. This increase was accompanied by an almost complete depletion of Put and Spd whereas Spm was not significantly affected (Fig. 12). The biochemical implications of these changes will be discussed elsewhere [38].

CONCLUSIONS

The described chromatographic procedure with dual detection, UV absorbance at 254 and 280 nm and fluorescence detection, after post-column derivatization with *o*-phthalaldehyde allowed the simultaneous determination of the SAM analogues and of the polyamines. As was shown, the different

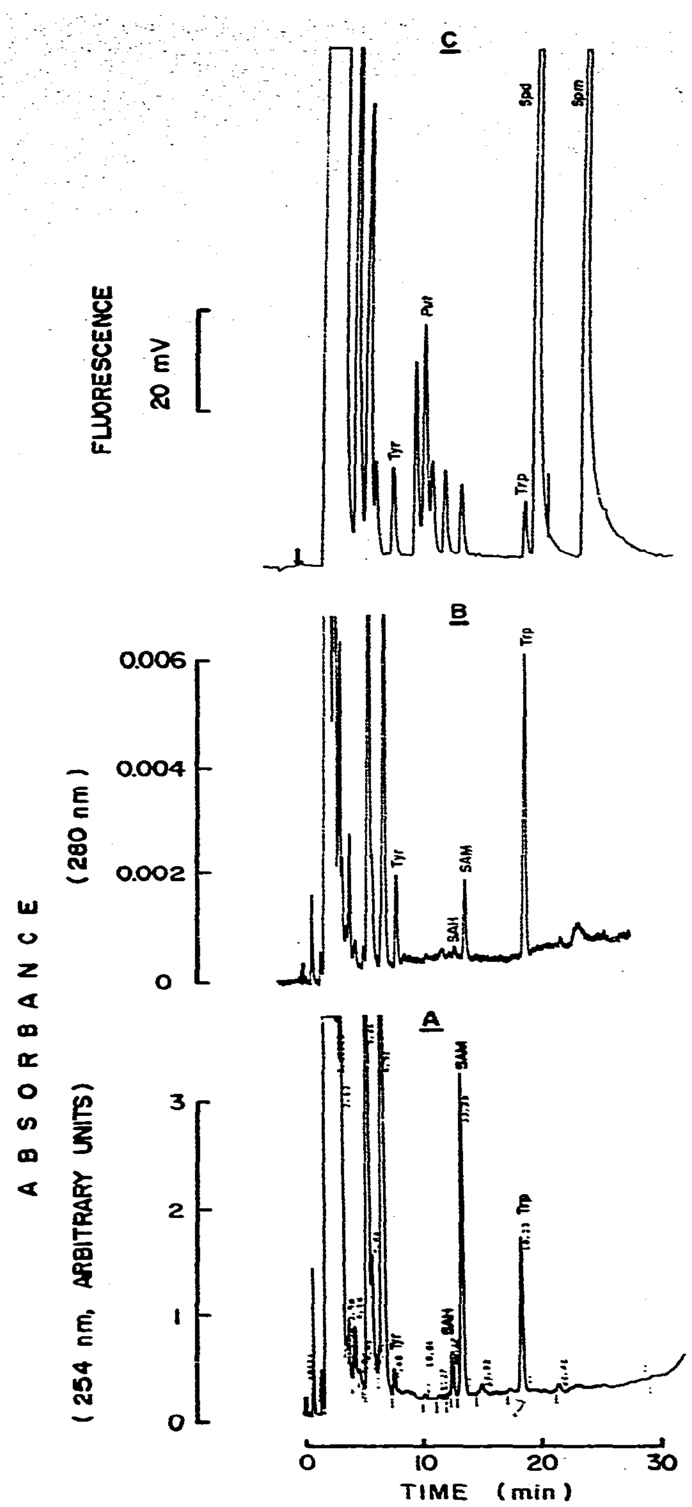


Fig. 11. Chromatograms obtained with UV detection at 254 nm (A) and 280 nm (B), and with fluorescence detection (C) of control HTC cells. A 50-μl aliquot of the extract prepared as described in the sample preparation section were applied to the column. Chromatographic conditions were similar to those of Fig. 7 and as described in the Methods section.

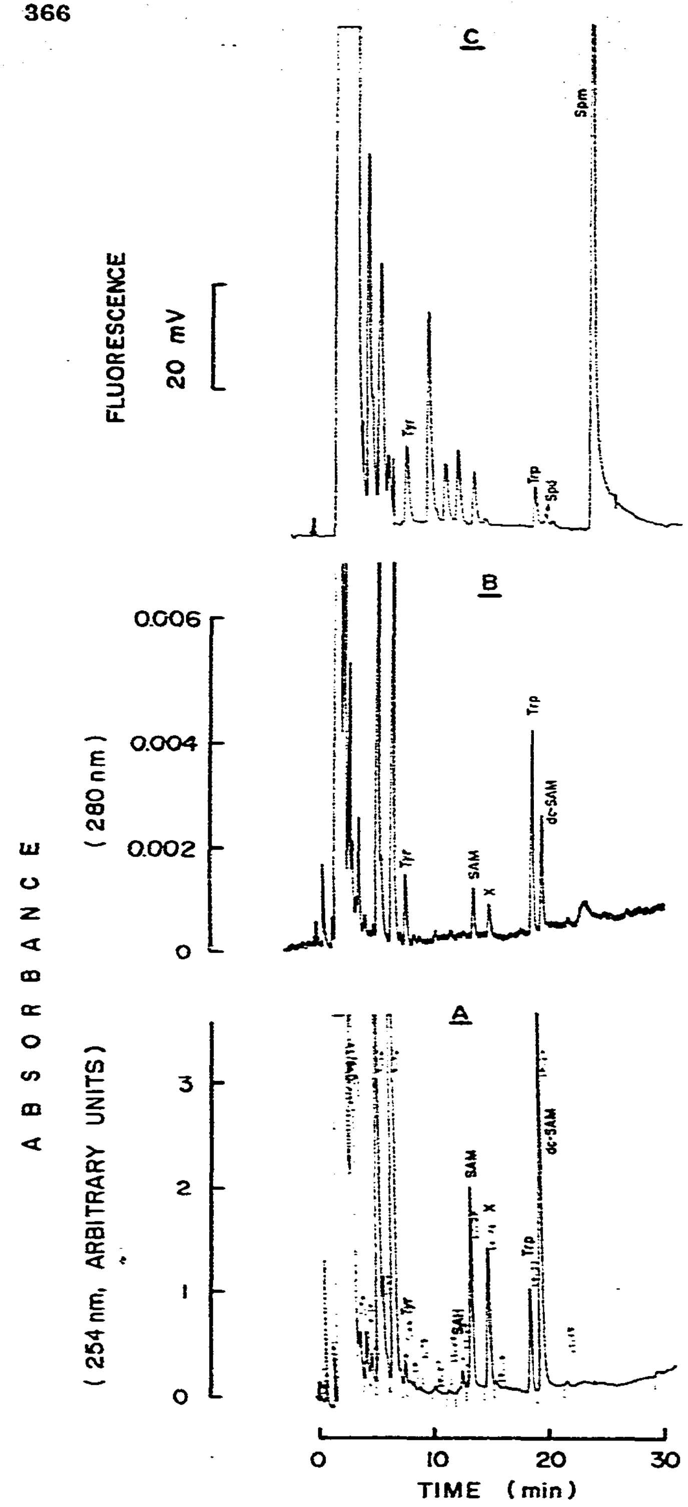


Fig. 12. Chromatograms obtained with UV detection at 254 nm (A) and 280 nm (B) and with fluorescence detection (C) of HTC cells treated for 48 h with 5 m*M* DFMO in the culture medium. For further details see Fig. 11.

chromatographic parameters, pH, ionic strength, temperature, OSA concentration, percentage of acetonitrile and type of gradient are strongly interrelated. Therefore it is obvious that the set of parameters that have been chosen is not the only one that would give the desired separation. We have used these properties to advantage when the capacity and efficacy of the columns deteriorate after extensive use, by changing one or the other of these parameters to obtain the necessary separation. The method, as described in this work, has been made semi-automatic with the use of an automatic injector and digital integrator and allows us to analyze up to 30 samples per day.

From the applications presented it is apparent that our reversed-phase ion-pair HPLC procedure with multiple detection for the simultaneous determination of SAM, SAH, dc-SAM and the polyamines in one single run is of major interest to study the biochemical consequences of the inhibition of ODC and of other enzymes involved in polyamine biosynthesis. Although the presence of dc-SAM had been shown previously in control tissues [7], our HPLC procedure showed for the first time that after treatment with an ODC inhibitor dc-SAM levels markedly increased. This increase seems to be related to the effect of the ODC inhibition in this given organ and may, in addition to the polyamine levels, be used as a marker of the inhibition of ODC [38].

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