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DETERMINATION OF POLYAMINES BY PRE-COLUMN DERIVATIZATION WITH *o*-PHTHALALDEHYDE AND ETHANETHIOL IN COMBINATION WITH REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The polyamines spermine, spermidine, putrescine and cadaverine were treated with *o*-phthalaldehyde and ethanethiol. After a reaction time of 90 sec, the stability of the derivatives was examined in the reaction medium, in an ethyl acetate extract and in different mobile phases used for high-performance liquid chromatography on reversed phase columns. The stability of the ethanethiol derivatives was improved compared to the 2-mercaptoethanol derivatives. Increasing concentrations of alcohol in the reaction medium increased the stability, but a significantly higher stability was obtained by including a simple and rapid extraction with ethyl acetate. With a mobile phase of N,N-dimethylcyclohexylamine (0.1 *M*) and phosphoric acid (0.2 *M*) in a 70–90% methanol gradient, decomposition on the column was minimized and a satisfactory resolution was obtained. A detection limit of less than 0.5 pmol of each polyamine was obtained by fluorescence detection.

INTRODUCTION

The physiological and biochemical significance of polyamines has necessitated the development of a series of analytical methods, which has been reviewed by Seiler[1,2]. High-performance liquid chromatography (HPLC) of the derivatives with *o*-phthalaldehyde (OPA) and 2-mercaptoethanol, combined with fluorescence detection, has largely been performed as post-column derivatization after separation on ion-exchange columns[3–5]. The limits of detection of the different polyamines have been reported to be 5–20 pmol[5].

The main reason for using post-column derivatization undoubtedly has been the limited stability of the OPA–mercaptoethanol derivatives. If the stability could be improved, rapid derivatization procedures in combination with the more efficient reversed-phase columns could make pre-column derivatization a realistic alternative to the post-column procedures.

When Simons and Johnson[6,7] studied the reactions between OPA, thiols and primary amines, the products with ethanethiol were found to be slightly more stable

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than the products with 2-mercaptoethanol (Fig. 1), due at least partially to the direct effect of the hydroxyl group of the mercaptoethanol residue on the conversion of the adduct (I) into the lactam (II). With amino acids no difference in the stability between the adducts of 2-mercaptoethanol and ethanethiol was reported[8]. The reaction with amino acids appeared to be complete in 2 min. In another study, which also utilized ethanethiol with amino acids, the reaction was reported to require at least 10 min for completion[9].

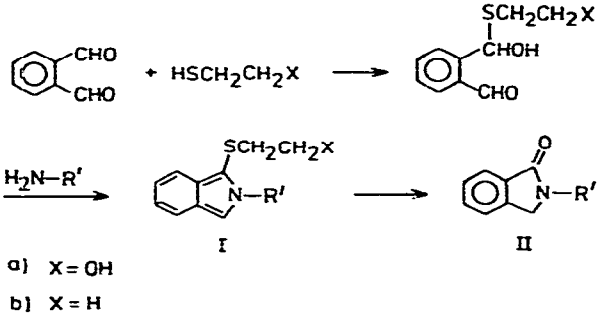

Fig. 1. The reaction of primary amines with OPA and thiols.

In pre-column derivatization procedures the yield of the adduct (I) at the moment of detection depends on the synthetic yield, the stability of the adduct in the reaction medium and the stability of the adduct during the chromatographic separation. The synthetic yield as well as the stability is known to be pH-dependent[6,7]. In the presence of an acid (Fig. 2), the protonated sulphide can undergo hydrolytic cleavage to an enol, which subsequently rearranges to the more stable keto form (II).

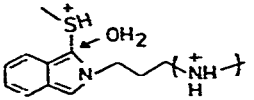

Fig. 2. Hydrolytic cleavage of the OPA derivatives in the presence of acids.

Since a hydroxyl group in the thiol moiety has been found to influence the stability of OPA adducts, and since the presence of thiol groups in the amino side-chain has resulted in non-fluorescent products[7], a similar interaction would be expected with an even better nucleophile, an amino group, in the amino side-chain. Thus, the two polyamines spermine and spermidine, containing unreacted secondary amino groups (Fig. 3), could be expected to be less stable than the polyamines with only derivatized primary amino groups.

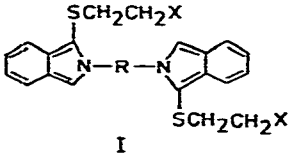

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Fig. 3. Derivatives of polyamines with OPA and thiols. a = Spermine (SPM), R = $(CH_2)_3NH(CH_2)_4NH(CH_2)_3$; b = spermidine (SPD), R = $(CH_2)_3NH(CH_2)_4$; c = putrescine (PUT), R = $(CH_2)_4$; d = cadaverine (CAD), R = $(CH_2)_5$; e = 1,6-hexanediamine (HDA), R = $(CH_2)_6$. The formation of the derivatives (I) is described by the general reaction scheme in Fig. 1.

The common use of 2-mercaptoethanol in post-column derivatization is mainly due to the high solubility of the reagent and the derivatives in aqueous solutions. With reversed phase columns, high solubility in aqueous solutions is not an urgent requirement. Thus, the use of ethanethiol for pre-column derivatization of polyamines ought further to be investigated. This report presents an examination of the stability of the derivatives of spermine, spermidine, putrescine, cadaverine and 1,6-hexanediamine (used as internal standard) with ethanethiol, compared to the derivatives with 2-mercaptoethanol. The stability in the reaction medium as well as in different mobile phases has been studied in order to evaluate the rate of degradation prior to detection. Since chromatographic methods have been used to measure the yields and since such methods contribute to the degradation processes, no rate constants have been measured. The degradation rates have been illustrated by measuring the amounts of non-degraded adducts after a specified time. Various reversed phase columns were examined since spermine and spermidine were suspected of causing problems with some reversed phases.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of Waters Assoc. Model 6000 A pumps, a Waters U6K valve loop injector, a Waters Model 660 gradient programmer and a Kontron SFM 23 LC spectrofluorometric detector. The excitation and emission bandwidths were 10 nm and 20 nm, respectively. The maximum excitation and emission wavelengths were 340 nm and 440 nm, respectively.

Chemicals

The polyamines and OPA were obtained from Sigma (St. Louis, MO, U.S.A.), N,N-dimethylcyclohexylamine, triethylamine, *n*-propanol, mercaptoethanol and boric acid (all puriss.) from Fluka (Buchs, Switzerland), ethyl acetate, sodium acetate and phosphoric acid (all p.a.) from E. Merck (Darmstadt, G.F.R.), acetic acid (99.5%) from J. T. Baker (Phillipsburg, NJ, U.S.A.), ethanol (absolute) from Vinmonopolet (Oslo, Norway), ethanethiol (p.a.) from Riedel-de Haen (Seelze-Hannover, G.F.R.), potassium hydroxide (p.a.) from Elektro-Kemiska (Bohus, Sweden) and methanol (HPLC) from Rathburn (Walkerburn, Great-Britain).

The water was distilled twice. The solvents were filtered through a 0.45-μm Millipore filter.

Derivatization with 2-mercaptoethanol

OPA and 2-mercaptoethanol were dissolved in absolute ethanol to concentrations of 10 mg/ml and 50 μl/ml, respectively. 0.4 *M* boric acid was adjusted with 2 *M* KOH to a pH of 9.4, 10.3 or 10.8. The best reproducibility was obtained at pH 10.8. Standard solutions of the polyamines in water were made up to concentrations of 10^{-4} *M*.

Nine parts of the borate buffer were added to one part of the OPA-mercaptoethanol solution. A 100-μl volume of this reagent was added to 25 μl of a polyamine solution and mixed in a Fision Scientific whirlimixer (30 sec). The reaction was complete after 75 sec, but better reproducibility was obtained by extending the reaction time to 90–120 sec.

Derivatization with ethanethiol

The procedure was equivalent to that above, only replacing 2-mercaptoethanol with ethanethiol. The pH 9.4 buffer appeared to give slightly higher peaks compared to the other buffers. The reagent was stored at +4°C. After storage for 25 days the yields of polyamine adducts were reduced by 10%.

For samples containing large amounts of other reactive components, a higher excess of OPA and ethanethiol is recommended. In the direct derivatization of 25 μl of serum, a ten-fold higher excess was required in order to ensure complete derivatization.

Columns

The properties of the following columns were examined: ODS-Hypersil (25 cm × 4.6 mm) and SAS-Hypersil (30 cm × 3.9 mm) from Shandon Southern (Runcorn, Great Britain); Spherosil/C_{18} Normatom (20 cm × 4.6 mm) from Prolabo (Paris, France) and Spherisorb-C_6 (15 cm × 4.6 mm) and Spherisorb-Phenyl (10 cm × 4.6 mm) from Phase Separations (Queensferry, Great Britain). All the packing materials had a particle diameter of 5 μm.

Mobile phases

All the columns above were examined in combination with the following mobile phases: 0.1 *M* acetic acid in 80% methanol (pH 2.9); 0.04 *M* acetic acid +0.01 *M* sodium acetate in 85% methanol (pH 5.7); 0.01 *M* acetic acid + 0.01 *M* sodium acetate in 85% methanol (pH 6.7); 0.01 *M* sodium acetate in 85% methanol (pH 8.6); 0.04 *M* acetic acid + 0.01 *M* sodium acetate + 2% tetrahydrofuran in 85% methanol and 0.01–0.04 *M* pyridine + 0.01 *M* acetic acid in 85% methanol (pH 5.3–5.5).

Based on the results obtained, the ODS-Hypersil column was selected to be used in further studies with the following mobile phases: 0.02–0.10 *M* triethylamine (TEA) in 85% methanol; 0.02–0.10 *M* triethylamine and 0.1–0.5 *M* phosphoric acid in 80% methanol; 0.1 *M* triethylamine and 0.5 *M* phosphoric acid in a 70–80% methanol gradient; 0.05–0.10 *M* N,N-dimethylcyclohexylamine (DCA) and 0.1–0.5 *M* phosphoric acid in 80% methanol or 0.1 *M* DCA and 0.2 *M* phosphoric acid in gradients with 60–90 (or 70–90)% methanol.

Stability in the derivatization medium

After an initial reaction time of 90–120 sec, 10-μl samples were injected on the column and the peak heights of the OPA adducts were determined. Further samples were withdrawn and injected at different times. The yield after a specified time interval was determined by the peak height, relative to the peak height after the initial reaction, in per cent.

The stability as a function of the content of alcohols was measured by adding alcohols prior to or after the initial reaction.

The effect of storage at low pH was determined by the addition of an equal volume of 0.5 *M* phosphoric acid to the medium after the initial reaction.

Stability in the mobile phases

A 100-μl volume of each mobile phase was added to 10 μl of the adduct solution directly after the initial reaction time of 90–120 sec. The amount of each

polyamine was 1 nmol. The peak height of each polyamine adduct was measured with time and compared to the peak height directly after addition of the mobile phase. Dilution effects were examined by using water blanks. The yields (in %) at a specific time were calculated as the amount relative to the amount directly after addition.

The relative precision (in %) was determined on the basis of three to five independent experiments.

Ethyl acetate extraction

After reaction for 90 sec, 125 µl of the reaction mixture containing 10–200 pmol of each polyamine were mixed for 1 min with 125 µl of ethyl acetate on the whirlimixer, then centrifuged for 1 min at 2000 *g*. 10-µl samples were withdrawn directly from the ethyl acetate phase. The quantitative transfer of derivatized polyamines to the ethyl acetate was controlled by injecting a sample from the aqueous phase.

RESULTS AND DISCUSSION

Stability of the mercaptoethanol derivatives in the reaction medium

At pH 10.8 the adduct yields were higher than at pH 9.4 (Table I). This was not due to a lower synthetic yield, but to a higher decomposition at pH 9.4. The decomposition rate was considered too rapid to assure a satisfactory reproducibility, even only with a reaction time of 1–2 min, 8 min after adding the reagents, more than 50 % of the spermine and spermidine adducts had decomposed (Table I). An example of the rapid degradation of spermine is shown in Fig. 4. The stability of the putrescine adduct was remarkably higher. The difference is believed to be caused by an intramolecular attack of the secondary amino group in spermine and spermidine (Fig. 5). This hypothesis is supported by the fact that lysine is known to give a lower yield of fluorescent OPA–mercaptoethanol adducts than other amino acids[10].

In order to examine the stability of significantly lower pH, an equal volume of 0.5 *M* phosphoric acid was added to the reaction products. To adjust for a dilution effect, measurements were also conducted with a water blank (Table I). With phosphoric acid the decomposition of putrescine was increased compared to spermine and spermidine (Table I).

TABLE I

YIELDS (%) OF THE OPA–MERCAPTOETHANOL ADDUCTS OF SPERMINE, SPERMIDINE AND PUTRESCINE AFTER 8 min IN THE DERIVATIZATION MEDIUM

The mobile phase for the analysis consisted of 0.1 *M* triethylamine and 0.5 *M* phosphoric acid in 70–80 % methanol.

Polyamine	*Derivatization pH*		*Water added to pH 10.8 medium after 2 min of reaction*	*H_3PO_4 added after 2 min. Final pH: 2.5*
	pH 9.4	*pH 10.8*		
Spermine	15 ± 4	40 ± 4	35 ± 5	25 ± 5
Spermidine	20 ± 6	45 ± 3	40 ± 4	30 ± 5
Putrescine	55 ± 9	70 ± 4	60 ± 4	< 10

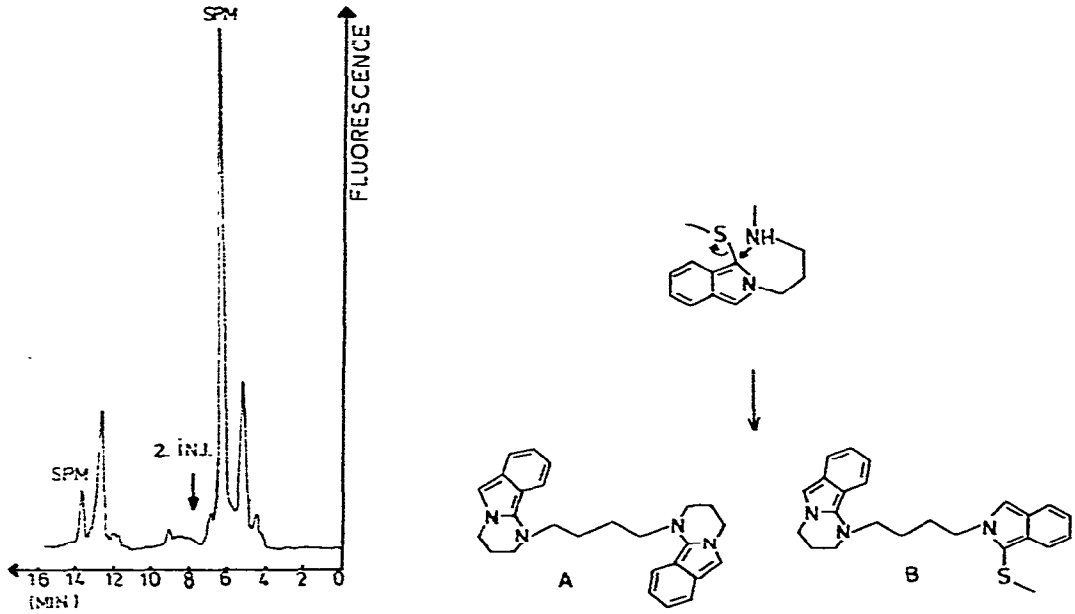

Fig. 4. Decomposition of the OPA–mercaptoethanol derivative of spermine. The first sample was injected after 2 min (at pH 9.4), the second sample after 8.45 min. The mobile phase consisted of 0.1 *M* TEA + 0.5 *M* H_3PO_4 in 70% methanol and the column was ODS-Hypersil (25 cm × 4.6 mm).

Fig. 5. Suggested intramolecular cleavage of the OPA derivatives of spermine (A) and spermidine (B) at high pH or in non-aqueous media.

At low pH the protonated secondary amino group cannot participate in an introamolecular nucleophilic attack. On the contrary, a protonated amino group in the side-chain would be expected to slow down the acid-catalyzed decomposition by reducing the rate of protonation of the vinyl sulphide (Fig. 2), thereby explaning the higher stability of spermine and spermidine compared to putrescine.

In agreement with the findings of Simons and Johnson[7], the stability was increased by increasing the amount of an alcohol in the solution. After 30 min there was only 10–15% decomposition (Table II). The use of *n*-propanol unfortunately resulted in interfering peaks in the chromatograms, probably from decomposition

TABLE II

YIELDS (%) OF THE OPA–MERCAPTOETHANOL ADDUCTS OF SPERMINE AND SPERMIDINE AS A FUNCTION OF THE CONTENT OF ALCOHOLS, MEASURED AFTER 30 min

The mobile phase for the analysis consisted of 0.1 *M* DCA and 0.2 *M* H_3PO_4 in 60–90% methanol. EtOH = Ethanol; PrOH = *n*-propanol.

Polyamine	*5% EtOH*	*50% EtOH*	*5% EtOH + 50% PrOH*
Spermine	20 ± 3	60 ± 3	85 ± 5
Spermidine	20 ± 3	70 ± 4	90 ± 5

products. High amounts of alcohols also caused precipitation in derivatization of serum samples. When the ethanol concentration was increased to more than 50%, the borate salts from the buffer started to precipitate.

Stability of the ethanethiol derivatives in the reaction medium

The stability of the derivatives was significantly improved by replacing 2-mercaptoethanol with ethanethiol. At pH 9.4, which now resulted in slightly higher peaks than at pH 10.8, 70–75% of the adducts was still intact after 20 min, compared to the 40–70% after 8 min with 2-mercaptoethanol. With the increased stability the effects of increasing concentrations of alcohols were better demonstrated after 30 min (Table III).

TABLE III

YIELDS (%) OF THE OPA–ETHANETHIOL ADDUCTS OF POLYAMINES AS A FUNCTION OF THE CONTENT OF ALCOHOLS, MEASURED AFTER 30 min

The mobile phase for the analysis consisted of 0.1 *M* DCA, 0.2 *M* H_3PO_4 and 70–90% methanol.

Polyamine	*Initial concn. of alcohol*		*2 min of reaction in 10% EtOH followed by 28 min in:*		
	40% PrOH	*50% EtOH*	*5% EtOH*	*55% EtOH*	*5% EtOH + 50% PrOH*
Spermine	68 ± 7	62 ± 6	63 ± 6	75 ± 7	84 ± 7
Spermidine	82 ± 8	84 ± 5	83 ± 7	84 ± 7	101 ± 5
Putrescine	91 ± 5	87 ± 4	89 ± 5	88 ± 5	105 ± 5
Cadaverine	82 ± 6	84 ± 5	86 ± 5	88 ± 5	107 ± 5
1,6-Hexanediamine	84 ± 7	86 ± 5	86 ± 5	89 ± 5	107 ± 5

Due to the higher stability of the ethanethiol derivatives, the stabilizing effect of higher alcohol concentrations was much less pronounced. Actually, a better detection limit was obtained by using the original reaction medium (with 10% ethanol), since higher sample volumes could be injected on the reversed phase columns with the more aqueous solutions.

Choice of columns and mobile phases

Two different C_{18} columns, with 7% and 20% carbon, respectively, two short-alkyl-chain columns and one phenyl column were examined with a set of mobile phases at pH values ranging from 2.9 to 8.6. A significantly better resolution was obtained on the two C_{18} columns, compared to the short alkyl chain and the phenyl columns. Little difference was observed between the two C_{18} columns. Since the ODS-Hypersil column was better packed and showed the highest efficiency, this column was used throughout this study and all the results described herein originate from this column. After 18 months of regular use, the properties of the old column were compared with a freshly prepared column of the same packing. No differences in the chromatographic behaviour of the polyamine adducts were observed.

All the chromatographic conditions were examined first with the mercaptoethanol derivatives, then adjusted to the ethanethiol derivatives. The choice of the additives to the mobile phase was found to be imperative for a satisfactory resolution.

With acetic acid or mixtures of acetic acid and sodium acetate in 85% methanol the spermine derivatives were totally retained. Similar behaviour has been observed with amines on this packing and other C_{18} packings, with the exception of μBondapak C_{18}, which gave less retention and better preak shape[11].

With 0.1 *M* TEA in 85% methanol all the polyamines were eluted, but the high pH (11) and the appearance of interfering peaks from other products of the derivatization made this solvent less attractive.

By adding phosphoric acid the pH was reduced and the desired resolution was obtained. All the polyamine derivatives with 2-mercaptoethanol were eluted as symmetrical peaks within 20 min by isocratic elution with 0.1 *M* TEA and 0.5 *M* phosphoric acid in 75% methanol. The ethanethiol derivatives were more strongly retained, but with 85% methanol all the polyamines were eluted with good resolution within 15 min (Fig. 6). With TEA and phosphoric acid the elution order was opposite to that with the acetate mobile phases, the two polyamines with secondary amino groups now eluting prior to the others. The detection limit was determined to be 3–4 pmol. A linear relationship between injected amount and the fluorescence response was found for all the polyamines in the 10–200 pmol range. The stability of the derivatives was still not regarded as satisfactory, possibly due to the low pH. The apparent pH was measured as 2.7. The concentration of phosphoric acid could, however, not be reduced, since this resulted in tailing peaks.

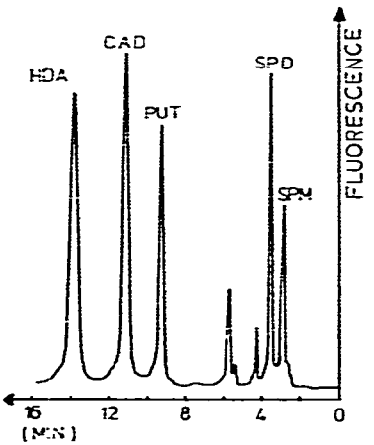

Fig. 6. Isocratic separation of OPA–ethanethiol derivatives of polyamines (100–200 pmol) with TEA (0.1 *M*) and phosphoric acid (0.5 *M*) in 85% methanol on ODS-Hypersil (25 cm × 4.6 mm), with a flow-rate of 1 ml/min.

By replacing TEA with DCA[10], a 0.2 *M* concentration of phosphoric acid and 0.1 *M* DCA was sufficient to obtain symmetrical peaks. Since material from samples of biological origin may accumulate on the columns during isocratic elution, the conditions for gradient elution were investigated. The mercaptoethanol derivatives eluted within 18 min with 60–90% methanol (Fig. 7) and the ethanethiol derivatives with 70–90% methanol within 27 min (Fig. 8). The retention time reproducibility for the ethanethiol derivatives was determined to be ±2.8% for spermine and ±6.8% for putrescine over a 3 month period. The apparent pH of the DCA mobile phase was

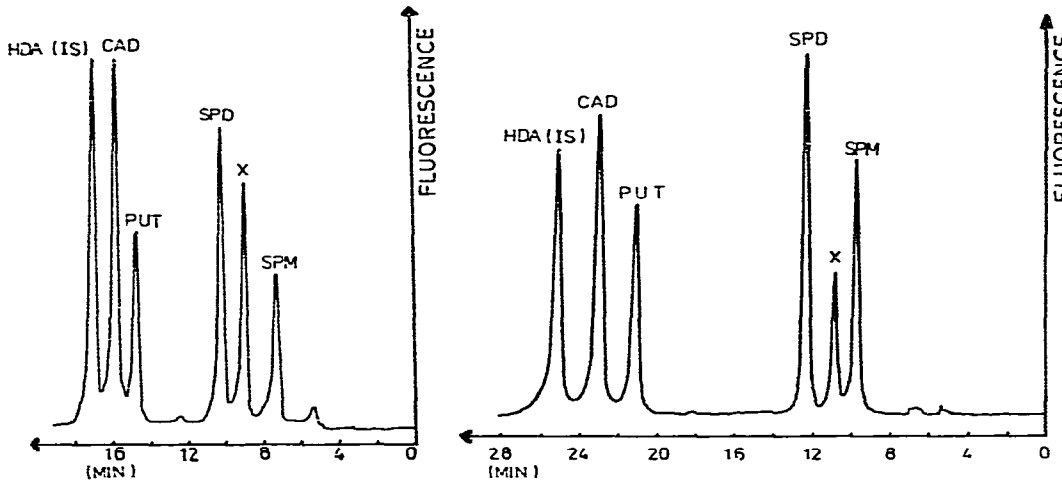

Fig. 7. Separation of OPA–mercaptoethanol derivatives of polyamines (200 pmol) with DCA (0.1 *M*) and phosphoric acid (0.2 *M*) in 60–90% methanol on ODS-Hypersil (25 cm × 4.6 mm), with a flow-rate of 1 ml/min and a gradient time of 15 min with gradient curve 5 of the Waters 660 gradient programmer.

Fig. 8. Separation of OPA–ethanethiol derivatives of polyamines (200 pmol) with DCA (0.1 *M*) and phosphoric acid (0.2 *M*) in 70–90% methanol on ODS-Hypersil (25 cm × 4.6 mm), with a flow-rate of 1 ml/min and a gradient time of 15 min with gradient curve 4 of the Waters 660 gradient programmer.

measured as 3.8. In this medium the stability and the peak height reproducibility were satisfactory. By using 1,6-hexanediamine as internal standard, a peak height precision better than ±2.6% was obtained. The detection limits were between 1 and 2 pmol.

Stability in the mobile phases

In order to determine the conditions for minimum degradation on the columns, stability tests in the mobile phases mentioned above were performed. With a total elution time of 18 min for the mercaptoethanol derivatives, Table IV shows that the DCA solvent was the only acceptable mobile phase. The higher stability of the

TABLE IV

YIELDS (%) OF THE OPA–MERCAPTOETHANOL ADDUCTS OF POLYAMINES DISSOLVED IN VARIOUS MOBILE PHASES AFTER AN INITIAL REACTION TIME OF 2 min

The equivalent mobile phases were used for the analysis. NaOAc = Sodium acetate; HOAc = acetic acid; MeOH = methanol. n.m. = Not measured.

Polyamide	*0.01 M NaOAc + 0.04 M HOAc in 85% MeOH after 8 min*	*0.1 M TEA in 85% MeOH after 8 min*	*0.1 M TEA + 0.5 M H_3PO_4 in 80% MeOH after 8 min*	*0.1 M DCA + 0.2 M H_3PO_4 in 80% MeOH after 30 min*
Spermine	n.m.	<30	14 ± 3	45 ± 5
Spermidine	40 ± 4	n.m.	28 ± 4	50 ± 5
Putrescine	65 ± 5	n.m.	73 ± 5	55 ± 6
Cadaverine	60 ± 5	n.m.	65 ± 6	55 ± 6

TABLE V

YIELDS (%) OF THE OPA–ETHANETHIOL ADDUCTS OF POLYAMINES DISSOLVED IN 0.1 *M* DCA, 0.2 *M* H_3PO_4 AND 80% METHANOL, AFTER AN INITIAL REACTION OF 2 min

The mobile phase of Table III was used for the analysis.

Polyamine	*8 min*	*30 min*
Spermine	89 ± 3	70 ± 4
Spermidine	89 ± 3	70 ± 4
Putrescine	90 ± 4	62 ± 3
Cadaverine	89 ± 3	60 ± 3
1,6-Hexanediamine	93 ± 5	62 ± 3

ethanethiol derivatives resulted in better precision of the peak height measurements (Table V).

Extraction into ethyl acetate

In order to examine whether the stability prior to the injection could be improved further without the not-so-attractive addition of ethanol, the ethanethiol derivatives were extracted into ethyl acetate after reaction for 90 sec. As is seen from Table VI, the stability was significantly improved. Even after more than 6 h, the yields varied between 31% (spermine) and 93% (cadaverine). The difference in the stability of the spermine and the spermidine adducts in this non-aqueous medium clearly demonstrates that the decomposition process is dominated by the intramolecular process. The assumed degradation product of spermine (Fig. 5) is non-fluorogenic, in contrast to the decomposition product of spermidine where the fluorescence is reduced but not absent. Samples from the ethyl acetate solution were injected directly on the reversed-phase column with no problems, and the peak height precision varied between ±2% and ±5% by using the internal standard method. The transfer of polyamine adducts to the ethyl acetate phase was quantitative (>95%).

An impurity (peak X) which could interfere with spermine at a large excess of reagents was quantitatively removed by the ethyl acetate extraction (Fig. 9). The impurity appeared to be a primary amine contaminant from one of the reagents of the derivatization, since reaction with 2-mercaptoethanol gave a similar peak and since the peak also appeared in blanks without the addition of polyamines. The detection

TABLE VI

YIELDS (%) OF THE OPA–ETHANETHIOL ADDUCTS OF POLYAMINES AFTER AN INITIAL REACTION TIME OF 90 SEC, FOLLOWED BY EXTRACTION INTO ETHYL ACETATE

The mobile phase in Table III was used for the analysis.

Polyamine	*2½ h*	*6½ h*	*23 h*
Spermine	80 ± 6	31 ± 3	<5
Spermidine	93 ± 4	83 ± 6	67 ± 7
Putrescine	96 ± 5	92 ± 5	90 ± 6
Cadaverine	97 ± 5	93 ± 5	85 ± 7
1,6-Hexanediamine	97 ± 5	92 ± 5	90 ± 6

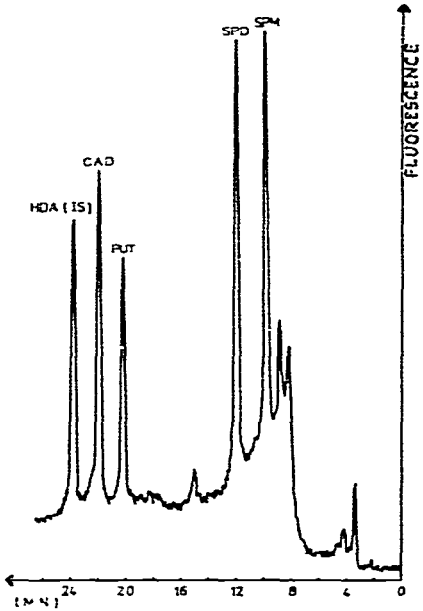

Fig. 9. Separation of OPA–ethanethiol derivatives of polyamines (4.5 pmol) after extraction and injection in ethyl acetate (10 μl), with chromatographic conditions as in Fig. 3. Note the disappearance of the impurity (peak X), compared to Fig. 8.

limit of the polyamines, using a 1:1 volume ratio of ethyl acetate and the derivatization solution, was less than 0.5 pmol of each polyamine. It was determined by injecting 10 μl or less of extracts after derivatization of 5 pM solutions of polyamines and was based on a signal/noise ratio of at least 3:1.

More concentrated extracts could be obtained by using a volume ratio of 1:3 between the ethyl acetate and the derivatization solution. Between the 1:1 and the 1:3 volume ratios, a linear relationship was obtained between the initial concentration and the detector response.

If mixtures of amino acids and polyamines were derivatized and chromatographed, the extraction process removed most of the amino acids which could interfere with the spermine peak. Preliminary experiments with direct derivatization of serum showed that a prepurification was required in order to determine normal polyamine levels in serum, due to the appearance of other interfering peaks.

With injection volumes of 10 μl or less, the injection of ethyl acetate extracts on the reversed phase columns caused no peak-broadening.

CONCLUSIONS

Ethanethiol gave mor stable derivatives with OPA and polyamines, compared to 2-mercaptoethanol. With a simple and rapid (<5 min) extraction into ethyl acetate, the stability was improved sufficiently to enable the use of automatic injectors overnight for extracts prepared during the day. Spermine should, however, be analyzed within 6 h after derivatization, since two thirds had then decomposed. The

extraction removed an interfering contaminant and also partially removed derivatized amino acids which would otherwise interfere with the spermine peak in biological samples.

If the extraction step is to be avoided, the injections could be performed after reaction for 90 sec or within approximately 30 min after derivatization, with satisfactory yields, good reproducibility and excellent resolution of the polyamines on C_{18} columns. The highest stability and the lowest detection limit was obtained by using a mobile phase consisting of 0.1 *M* DCA and 0.2 *M* phosphoric acid in 70–90% methanol.

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