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Journal of Chromatography B, 759 (2001) 285–297

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

o-Phthalaldehyde–*N*-acetylcysteine polyamine derivatives: formation and stability in solution and in C_{18} supports

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Received 11 September 2000; received in revised form 4 May 2001; accepted 4 May 2001

Abstract

A comparative study of different derivatization procedures has been performed in order to improve the stability of the reaction products *o*-phthalaldehyde–*N*-acetylcysteine (OPA–NAC) polyamines. Procedures such as solution derivatization, solution derivatization followed by retention on a packing support, derivatization on different packing supports and on-column derivatization, have been optimized and compared. The degradation rate constant (*k*) of the derivative was dependent on the procedure used and on the analyte. For the spermine (the most unstable isoindol tested) *k* was $8\pm2\times10^{-2}$ min^{-1} in solution versus $7.7\pm1.1\times10^{-4}$ min^{-1} on the (C_{18}) solid support. The results obtained showed that forming the derivative on the packing support (C_{18}) gave the best results following this procedure: conditioning the cartridges with borate buffer (1 ml, 0.5 *M*, pH 8), retention of the analyte, addition of 0.8 ml of OPA–NAC reagent, 0.2 ml borate buffer 0.8 M (pH 8) and elution of the isoindol with 3 ml of MeOH–borate buffer (9:1). The different derivatization procedures have been used to study the stability of the reaction products OPA–NAC polyamines formed in urine matrix using spermine as model compound. Similar results were obtained for standard solutions and urine samples.

Keywords: Derivatization, LC; *o*-Phthalaldehyde–*N*-acetylcysteine; Polyamines

1. Introduction

Many compounds, such as simple aliphatic amines or alcohols, lack a chromophore and cannot be readily analysed by high-performance liquid chromatography (HPLC) using spectrophotometric or fluorescence detection. This problem often can be overcome by derivatization to introduce a chromophore or fluorophore.

o-Phthalaldehyde (OPA), in combination with a thiol compound such as 2-mercaptoethanol (MCE), is widely used for the pre-chromatographic fluorescent derivatization of amino compounds, for instance amino acids or biogenic amines (polyamines). However OPA derivatives have the disadvantage of limited stability, being dependent, among other characteristics, upon the steric hindrance around the NH_2 group or the kind of thiol used. This is especially true for amines also containing secondary amino groups, such as spermine [1]. Some attempts have been made to improve the stability of the product OPA–amino acids. The use of bulky thiols

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00236-5

such as *N*-acetylcysteine (NAC) or 3-mercaptopropionic acid (MPA), provides more stable isoindols, compared to those formed with OPA–MCE [2]. Other alternatives, such as the addition of sodium dodecyl sulphate (SDS) or phosphate buffer (pH 4), or the extraction of the reaction product, have been demonstrated insufficient for stabilising the OPA-derivatives. Recently, some papers have been published in reference to the stability of the OPA–NAC reagent and their amino acid derivatives [3,4] in aqueous solution, and they confirm that the lack of stability of these compounds depends on the analyte and the reaction conditions.

Many articles have been published about the application of this reagent to amine determination [5], and in order to afford reproducible results automated or on-column systems for the precolumn derivatization have been developed [6,7]. Saito et al. [8] have carried out a kinetic study of the stability of the OPA–spermine (Spm) fluorophore formed by an on-column method, with a mobile phase containing OPA–NAC reagent, coupled with a column-switching system. They found that the OPA–Spm fluorophore formed by the on-column method was more stable than that formed by a conventional pre-column method. Recently, an automated procedure has been developed to determine polyamines with OPA in different biological samples [9]. The proposed procedure required off-line extraction followed by on-line derivatization, and has some limitations for determination in real samples such as urine. Nevertheless, the special spectrophotometric and fluorimetric characteristics and the stability of the OPA–NAC reagent and OPA–NAC-amine isoindol have not been investigated in detail in the literature.

Thus, this paper describes a kinetic spectrophotometric and fluorimetric study of the stability of the OPA–NAC reagent and OPA–NAC-amine derivative by using different derivatization procedures such as solution derivatization, solution derivatization followed by retention on solid supports, derivatization on solid supports and on-column derivatization. Derivatization on commercial solid support is a recent methodology proposed by this research group as a quantitative procedure for amine determination. Off-line [10–12] and on-line procedures [13–15] on solid-phase supports by using different derivatization reagents (dansyl chloride, 1,2-naphtoquinone 4-sulphonate, etc.) have been developed. In this methodology, a commercial Si–OH-modified packing material is used instead of polymeric reagent specially prepared for solid-phase derivatization. The cartridges are used to purify the sample, to concentrate the analytes and to perform the derivatization. Some applications have been described for determination of amino compounds in urine samples, such as amphetamines, ephedrines or polyamines.

In this paper, the different derivatization procedures have been studied and compared. In the best conditions, the stability of spermine in urine samples has been studied, in order to determine the utility of these approaches to routine quantification. The amines assayed have been putrescine, spermine and spermidine, which are involved in the processes of cell proliferation, cell differentiation and malignant transformation [16,17]. Probably patients with many types of cancer have enhanced urinary excretion of polyamines [18–21]. Furthermore, it has been found that the determination of biogenic amines is a useful quality parameter related to the decomposition of food matrix, such as in fish [22], soy sauce [23] or milk [24]. Due to their potential usefulness as biochemical markers, many methods have been developed for their separation and determination in various biological samples, for example: ion-exchange chromatography [25,26], capillary zone electrophoresis [27,28], thin-layer chromatography [29], gas chromatography [21] and specially high-performance liquid chromatography (HPLC) [30–39].

2. Experimental

2.1. Apparatus

All spectrophotometric measurements were done on a Hewlett-Packard (Palo Alto, CA, USA) HP 8453 diode-array spectrophotometer furnished with quartz cells with a 1-cm pathlength.

Fluorescence was monitored using an Hitachi fluorescence spectrophotometer (Hitachi, Japan) model F-4500 furnished with quartz cells with a 1-cm pathlength.

The chromatographic system used consisted of a quaternary pump equipped with an automatic injector (1050 series) (Hewlett-Packard, Palo Alto, CA) with

a 100-μl sample loop injector, and a high-pressure six-port valve (Rheodyne model 7000). A diode-array detector (Hewlett-Packard, 1040 series) and fluorescence detector (Hewlett-Packard, 1050 Series) were coupled in series and linked to a data system recorder (Hewlett-Packard, HPLC Chem Station) for data acquisition and storage. The chromatographic signal was monitored at 247 nm for UV detection, with excitation at 333 nm and emission at 440 nm for fluorescence. All assays were carried out at room temperature.

2.2. Reagents

All chemicals were of analytical reagent grade unless stated otherwise. Putrescine dihydrochloride, spermine tetrahydrochloride and spermidine trihydrochloride were obtained from Sigma (St. Louis, MO).

N-Acetylcysteine (NAC) was obtained from Aldrich-Chemie (Steinheim, Germany). Sodium hydrogen carbonate (Probus, Badalona, Spain), boric acid, hydrochloric acid and sodium hydroxide (Panreac, Barcelona, Spain) were also used. Methanol (Merck, Darmstadt, Germany) and acetonitrile (Baker, Deventer, Holland) were of HPLC grade. All solutions were prepared with ultrapure water from a Nanopure II system (Sybron, Barnstead).

Bond Elut C_2, C_8 and C_{18} 200-mg extraction columns were from Varian (Harbor City, CA, USA). For urine samples Bond Elut Certified (3 cm^3 mg^{-1}) and Bond Elut PPL (6 cm^3 (500 mg)$^{-1}$) cartridges were also used.

2.3. Standard solutions

Stock standard solutions of amine (200 mg l^{-1}) and NAC (0.04 mol l^{-1}) were prepared by dissolving the pure compound in water. Stock standard solutions of OPA (0.01 mol l^{-1}) were prepared by dissolving the pure compound in water containing 15% methanol (MeOH). All solutions were stored in the dark at 4°C. Working standard solutions at different concentrations were prepared from the stock standard solutions. Borate buffer (0.5 mol l^{-1}), pH 8, was prepared by dissolving an adequate amount of boric acid in water and then adjusting the pH with NaOH.

Concerning the stability characteristics of OPA–NAC reagent, UV–Vis measurements were performed on the stored reagent solutions (all solutions were stored at ~4°C for several periods of time). Solutions stored in the dark, at 4°C and in absence of borate buffer were stable for at least 8 days, while longer periods of time presented a variation coefficient of 5.3%, which agrees with Refs. [3,4]. At room temperature and in the presence of light, this solution suffered a decomposition process and a new spectrophotometric band at 440 nm appeared.

2.4. Urine samples

Untreated urine samples were spiked with polyamines solution at concentration level of μg ml^{-1}.

2.5. Solution derivatization (procedure I)

A 1.8-ml volume of 3.6×10^{-3} mol l^{-1} OPA–NAC reagent containing 6.7% MeOH was mixed with 100 μl of amine working standard solution and 100 μl of borate buffer (0.5 mol l^{-1}, pH 8.0) for the spectrophotometric study. The absorbance at 333 and 297 nm was recorded as a function of time starting at 20 s after the addition of the borate buffer solution, at 30-s intervals up to 170 s.

For the fluorimetric study the concentration of the OPA–NAC reagent was ten times lower than for the spectrophotometric procedure. The amine concentration was also lower. The fluorescence intensity, $\lambda_{excitation}=330$ nm and $\lambda_{emission}=440$ nm, was recorded as a function of time starting at 20 s after the addition of the borate buffer solution, at 30-s intervals up to 170 s.

Urine samples (1–2 ml) cannot be processed directly and a clean-up step with C_{18} cartridges was employed.

2.6. Solution derivatization followed by retention on packing supports (procedure II)

Solid-phase extraction cartridges were conditioned by drawing 1.0 ml of methanol, followed by 1.0 ml of borate buffer 0.5 mol l^{-1} (pH 8). After the required time (depending on the amine) the mixture formed by 0.8 ml of 7.4×10^{-3} mol l^{-1} OPA–NAC

reagent and 1 ml of amine standard solution was transferred to the cartridges. After a fixed time (20 s or more usually 2 min) the derivative was desorbed with 3 ml of MeOH–borate buffer (9:1). Following this, absorbance measurement was made at the wavelengths as described above.

For urine samples, 0.8 ml of 7.4×10^{-3} mol l^{-1} OPA–NAC reagent, 0.05–1 ml of urine (blank or fortified) and 0.2 ml of borate buffer (0.8 mol l^{-1}, pH 8) were mixed. The formed derivatives were retained on C_{18} cartridges and eluted following the procedure described for standard solutions. In order to eliminate the reagent and reagent products, a washing step with 3 ml MeCN was included.

2.7. On column derivatization (procedure III)

A 25-μl sample of the mixture (0.8 ml OPA–NAC, 0.2 ml water and 2 ml standard amine solution) was injected into a chromatographic system. The reaction took place in the coil after mixing with the mobile phase containing borate buffer. An ODS-Hypersil (125×4 mm I.D., 5 μm; Hewlett-Packard) column was used as an analytical column for separation of the derivatives. A methanol–borate buffer (0.05 *M*, pH 9) gradient elution mode was used (20:80 at $t=0$ and 60:40 at $t=2$ min), at flow-rate of 1 ml min^{-1}. All the solvents were filtered with a 0.45-μm nylon membrane (Teknokroma, Barcelona, Spain) and degassed with helium before use.

Aliquots of urine sample were directly injected into the chromatographic system and derivatized according to the procedure described above.

2.8. Derivatization on packing supports (solid-phase extraction cartridges) (procedure IV)

The solid-phase extraction cartridges were conditioned by drawing 1.0 ml of methanol, followed by 1.0 ml of borate buffer 0.5 mol l^{-1} (pH 8). Two different procedures were applied for standard solutions.

(1) Option A: A 2-ml sample of amine working standard solution was transferred to the cartridges. After this, 0.8 ml of OPA–NAC reagent (7.4×10^{-3} mol l^{-1}), and 0.2 ml of borate buffer (0.8 mol l^{-1}, pH 8) were transferred to the cartridges. After 3 min, the derivative formed was desorbed from the C_{18} cartridges with 3 ml of MeOH–borate buffer (9:1), pH 10.2.

(2) Option B: A 0.8-ml sample of OPA–NAC reagent (7.4×10^{-3} mol l^{-1}) was transferred to the cartridges. After this, 2 ml of amine working standard solution and 0.2 ml of borate buffer (0.8 mol l^{-1}, pH 8) were transferred to the cartridges. After 3 min, the derivatives formed were desorbed from the C_{18} cartridges with 3 ml of MeOH–borate buffer (9:1). The absorbance measurement was made as described above.

The urine samples were processed following option A. A 2-ml aliquot of urine was passed through C_{18} cartridges previously conditioned. In order to clean-up the sample several solvents were assayed: water, MeCN, water:MeCN (1:1), MeOH, and MeCN:BO_2^- (0.5 *M*) (9:1), pH 10.2. The derivatization was performed into the column following the procedure described for standard solutions.

3. Results and discussion

3.1. Standard solutions

3.1.1. Effect of reaction parameters on solution derivatization: kinetic data

Stability studies of the OPA–NAC-polyamine derivatives have been carried out by spectrophotometric and fluorimetric detection. The analytical signals of OPA, NAC, OPA–NAC and OPA–NAC-polyamine solutions were recorded as a function of time. For all the assayed analytes the results showed maximum absorbance values at 333 nm for OPA–NAC-polyamine and 297 nm for OPA–NAC reagent, and maximum fluorescence values at excitation and emission wavelengths of 330 and 440 nm. These values were in accordance with the wavelengths obtained by other 1-alkylthio-2-alkyl isoindoles [8]. The OPA–NAC-amine derivatives formed in the presence of putrescine and spermidine were rather stable until 10 and 6 min, respectively, degradation not being a major problem in these determinations.

However, the solutions of OPA–NAC-spermine isoindol suffered a decomposition process that decreased the absorbance of the wide band at 333 nm.

In this case, the reaction can be followed and the equation $\ln R/R_0 = -kt$ can be applied, R (the response at any time t) being equal to ϵlc where ϵ is molar absorptivity at 333 nm, l is the pathlength, and c is the analyte concentration, and R_0 is the initial response. The derivatization process fitted to this equation ($\ln R = \ln R_0 - kt$) can be divided into two steps. For the derivative, a first stage up to ~1 min where the reaction rate increases, and a second stage corresponding to a decomposition process up to 25 min. For the reagent, the reaction rate increases up to 6 min and is followed by a stabilization process up to 25 min. The degradation rate constant ($k = 8 \pm 2 \times 10^{-2}$ min^{-1}) ($n = 7$) was calculated from the slope of the above mentioned curve for spermine concentration ranging from 0.77 to 25 μg ml^{-1}, and time between 1 and 25 min. This fact confirms that the dependence is first order as reported Saito et al. [8]. When fluorescence measurements were performed the constant value was ten times higher due to the fact that the range concentration was ten times lower.

Fig. 1 shows the chromatograms corresponding to the injection of the blank reagent and polyamine derivatives at different reaction times. The spermine peak is only observed for short times. The other peaks appear also in the blank solutions.

The intensity of the absorbance depends on the molar ratio OPA–NAC. A significant decrease in the response was observed using a molar ratio different from (1:1). This behavior may be caused by the limited stability of the OPA reagent. Saito et al. [8] indicated that an excess of OPA in the derivatization step served to catalyse the degradation of the isoindole derivative. An OPA–NAC (1:1) molar ratio was found to be the most suitable. Similar results were obtained for all the studied polyamines.

The optimum pH was determined by derivatizing spermine with OPA–NAC at pH values ranging from 6.7 to 10.3 using borate, carbonate and phosphate buffers (Fig. 2a). From the results obtained, carbonate and borate buffers at pH 8 appear to be the optimum. At lower and higher pH the reaction rate increased. Phosphate buffer was not adequate because it causes a quick decomposition of the derivative. However, the effect of the borate buffer concentration was evaluated (Fig. 2b). The absorbance increased when the borate concentration increased. From this study a borate buffer of 0.025 mol l^{-1} was chosen as the working buffer concentration, since the results obtained showed a saturation area.

The influence of the percentage of MeOH and OPA–NAC concentrations on the reaction rate using a three-dimensional image plot were studied (Fig. 2c). From this plot it was deduced that a MeOH (%) ranging between 4 and 6.5% and an excess of reagent between 20- and 60-fold was adequate.

The conditions of maximum sensibility and low degradation rate constant were selected as optimum conditions (Table 1). The OPA–NAC ratio, pH buffer and MeOH (%) proposed by Molnár-Perl et al. [3,4] for amino acids derivatization with OPA–NAC are 1:3, 9.3 and 100%, respectively. These conditions are different from those obtained in this work for polyamines (Table 1), however, the reagent excess optimized is the same. The reaction time for polyamines is markedly shorter than for amino acids (7–28 min). The stability of the isoindols (polyamines or amino acids) depends on the analyte.

The equations of the calibration graphs are shown in Table 2 (procedure I). These results indicate that the precision and the accuracy of this method are satisfactory.

Statistical analysis of the analytical signal for the reagent and the reaction product prepared on different days and taking into account several variables such as pH, methanol (%), and reagent excess, allowed evaluation of the robustness of the procedure. The results are shown in a Shewhart plot (Fig. 3) ranged from ($x \pm 2s$). The average value (x) and the standard deviation (s) of the standard or blank signal obtained the 1st day give the warning limits. All the results obtained over 36 days are inside the warning limits. The variation coefficient was 6%, the usual value for this kind of procedure.

3.1.2. Solution derivatization followed by retention on packing supports

By working at the selected conditions (Table 1), the reaction products were retained on C_{18} cartridges. The percentage of derivative retained, considering the analytical signal of the derivatized solution at 1 min as 100%, were 72 ± 5, 92 ± 2, 90 ± 1 and 90 ± 2 ($n = 3$), for the blank reagent, spermine, spermidine and putrescine, respectively. From these results we can conclude that the retention of the

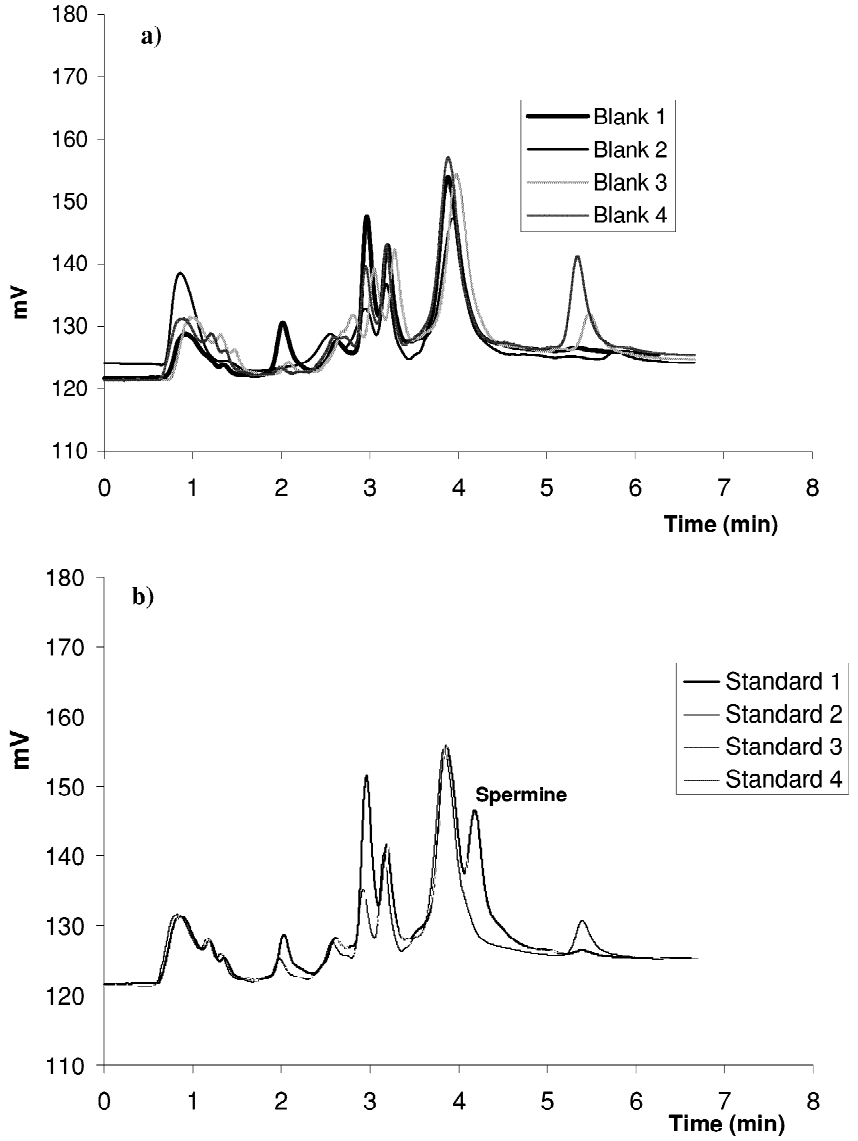

Fig. 1. Chromatograms corresponding to solution derivatization of the blank reagent and the spermine standard solution (4.49 mg l^{-1}) at different reaction times: (1) 1 min; (2) 8 min; (3) 20 min; (4) 30 min.

isoindol of the polyamines into the C_{18} cartridges was quantitative.

Different Bond Elut columns (C_{18}, C_8 and C_2) were tested and the best results were obtained using the packing $C_{18.}$.

In order to elute the reaction products, several solvents were used and the recoveries obtained are shown in Fig. 4. As can be seen, the reagent was nearly completely eluted using acetonitrile (MeCN) and MeCN with borate buffer. For the OPA–NAC-polyamine derivative good results were obtained using MeOH–borate buffer (9:1). The pH study showed that the best recoveries were obtained around pH 10. Study of the volume required to obtain the maximum recovery indicates that 3 ml MeOH–borate buffer (9:1) at pH 10 was appropriate because

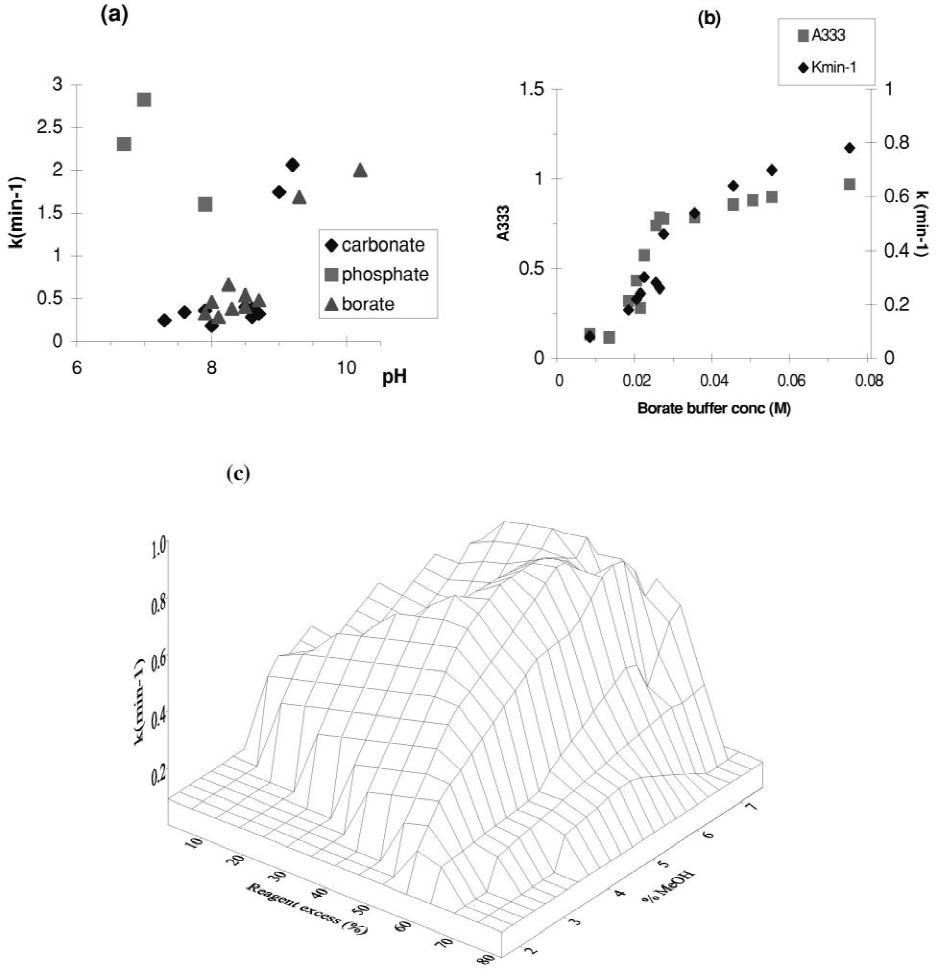

Fig. 2. Effect of several parameters on the analytical signal (Absorbance) and/or on the reaction rate (*k* is kinetic constant). (a) Effect of pH and buffer on the analytical signal of OPA–NAC and OPA–NAC-spermine; (b) effect of the borate buffer concentration; (c) effect of MeOH (%) and OPA:NAC concentration on the reaction rate.

Table 1
Working range and optimal value obtained for the different variables studied in solution derivatization

Conditions	Working range	Optimal value
OPA–NAC	1:1–2:1–2:1	1:1
pH Buffer	6–10	8.0
Buffer concentration (*M*)	0–0.08	0.025
% MeOH	2.7–7.5	4–6.5
Reagent excess (%)	0–80	20–60
Reaction time (s)	Spermine, 0–25 min	60 s
	Spermidine, 0–6 min	180 s
	Putrescine, 3–10 min	180 s

the results obtained for the first fraction were near the best recovery.

This methodology allows inclusion of a washing step after derivatization, especially when real samples need to be processed, in order to elute the reagent or reagent products different to the analyte products from the support. No significant differences were observed in the analytical signal by cleaning the derivative products retained on the support with 3 ml of MeCN.

The solid supports can be regenerated by passing through 1 ml of MeCN and 1 ml of MeOH.

Table 2
Analytical properties corresponding to the determination of several polyamines by using different procedures and different analytical signals

Procedure[a]	Analyte	Signal	$a \pm s_a$	$b \pm s_b$	r^2	$s_{y/x}$	LOD (ppm)	LC	Concentration range (ppm)
I	Spermine	A	0.0249±0.0055	0.0433±0.0003	0.9999	0.0079	0.3	0.9	0.3–25
		I	60±34	744±21	0.998	55.6	0.14	0.5	0.5–3
	Spermidine	A	0.01415±0.0003	$0.06139 \pm 2 \times 10^{-5}$	0.999	4×10^{-4}	0.074	0.25	0.25–25
		I	550±40	1470±30	0.999	57.09	0.077	0.26	0.26–2.1
	Putrescine	A	0.069±0.05	0.152±0.009	0.9998	7.6×10^{-3}	0.2	0.7	0.7–11
		I	530±25	5400±500	0.998	122.6	0.014	0.046	0.05–0.3
II	Spermine	A	0.022±0.015	0.0448±0.0016	0.998	0.017	0.94	3.11	3.11–40
	Spermidine	A	0.09±0.01	0.084±0.002	0.999	0.016	0.35	1.19	1.19–6
	Putrescine	A	0.01±0.02	0.132±0.005	0.998	0.02	0.45	1.51	1.51–11
III	Spermine	I	−52.0±65	180.4±8.5	0.996	97.95	0.05	0.2	0.2–15
IV	Spermine	A	0.164±0.001	$0.0156 \pm 2 \times 10^{-4}$	0.999	1.4×10^{-3}	0.29	0.97	0.97–10.4
	Putrescine	A	0.01±0.01	0.060±0.003	0.999	0.01	0.5	1.7	1.7–11

For more details, see Experimental section. A, absorbance; I, fluorescence.
[a] Procedure I: solution derivatization. Procedure II: solution derivatization followed by retention on packing supports. Procedure III: derivatization on column. Procedure IV: derivatization on solid support.

We found that the apparent rate constant OPA–NAC-spermine derivative onto the solid support was $(7.7 \pm 1.1) \times 10^{-4}$ min^{-1} after a certain period of time from 5 to 30 min before the elution. These results were in agreement with those obtained by Saito et al. [8].

These results indicated that the retention of the reaction product onto the solid support does not affect the reaction rate, and the low degradation rate of the products into the column allows increasing reaction product stability. Moreover, the degradation rate constant of the reaction product after elution was $(10 \pm 3) \times 10^{-3}$ min^{-1}, lower than that obtained for solution derivatization.

The analytical properties obtained by working at

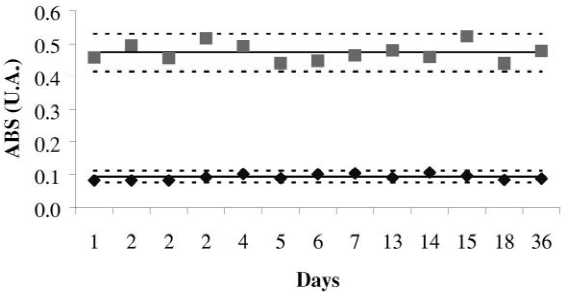

Fig. 3. Shewhart plot for the absorbance between days of the spermine derivative (▨) and the blank reagent (♦), ranged from $x \pm 2s$. Conditions: solution derivatization (for more details see Experimental section).

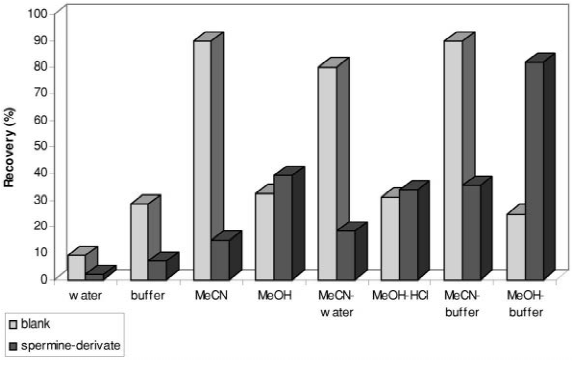

Fig. 4. Recoveries (%) obtained by using different elution solvent. Conditions: retention on solid support (for more details see Experimental section).

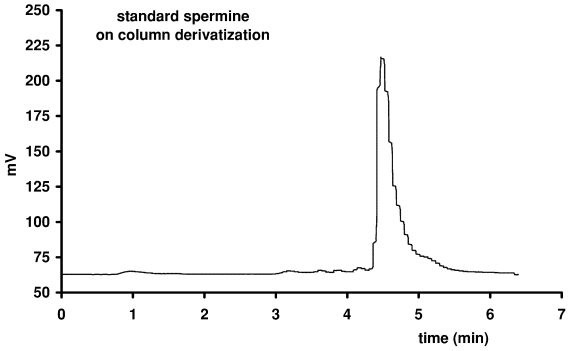

Fig. 5. Chromatogram corresponding to the OPA–NAC-spermine derivative (15 mg l^{-1}) obtained by on-column derivatization (procedure III).

Table 3
A comparative sensitivity study of the different procedures in which spermine has been used as analyte

	UV-visible	Chromatography
Procedure I	0.0433 ($n=4$)	66 ($n=1$)
Procedure II	0.0448 ($n=4$)	142 ($n=3$)
Procedure III		180 ($n=1$)
Procedure IV	0.0156 ($n=4$)	

Results were obtained from the slope of the calibration graphs with standards. Procedure I: solution derivatization. Procedure II: solution derivatization followed by retention on solid support. Procedure III: derivatization on column. Procedure IV: derivatization on solid support.

the optimized conditions are shown in Table 2 (procedure II).

3.1.3. On column derivatization (procedure III)

Due to the low reactivity between OPA–NAC and polyamines in the absence of buffer, these reagents were mixed before injection. The reaction took place in the chromatographic system in the presence of borate buffer in the mobile phase. In Fig. 5 is shown the chromatogram obtained for a spermine standard solution (15 ppm). The analytical signal obtained was higher than that obtained by using solution derivatization with and without retention into the solid support, as can be seen in Table 3.

3.1.4. Derivatization on packing supports (procedure IV)

According to our methodology [10–12] the steps and the optimal conditions for this procedure are indicated in Table 4.

The analytical parameters obtained by using this procedure are shown in Table 2 (procedure IV). In this procedure two different options were assayed (options A and B, Table 4). No significant differences were observed between the two options assayed. Thus, option A was chosen for further studies to allow the inclusion of a clean-up step before the reagent addition when required.

If we compare the results obtained with the other procedures previously described, the sensitivity obtained is lower (Table 3). This property can be

Table 4
Schedule of the different steps involved in the derivatization of polyamines on solid supports

Step	Option A	Option B
1) Support conditioning	1 ml MeOH 1 ml Borate buffer 0.5 *M*, pH 8	Idem
2) Analyte retention	2 ml Standard solution	Step 3
3) Reagent addition	0.8 ml (OPA:NAC, 1:1) in 15% MeOH 0.2 ml Buffer (0.8 *M*, pH 8)	Step 2
4) Reaction time (min)	3 min	Idem
5) Elution (ml)	3 ml (MeOH:borate buffer, pH 9.5, 9:1)	Idem

improved by increasing the reaction time in the column or the reagent concentration; however, volumes higher than 1 ml reagent solution can cause the analyte elution.

By using this procedure the stability of the reaction product increases. The reaction products formed onto the column and later eluted are stable for longer than 360 s.

3.2. Study of urine samples

3.2.1. Solution derivatization

Direct solution derivatization of polyamines in urine samples is not possible due to the high analytical signal obtained for the blank urine, because of the complexity of the matrix, and the quick degradation of the OPA–polyamine derivatives after their formation. Thus, it has become common practice to extract the interferences before or after the derivatization and before the analytical measurement in order to increase selectivity or even to employ automated systems to increase the stability.

According to previous work [12] by our research group, the amines were retained on a solid support (C_{18}) and several solvents were assayed to elute the matrix from the cartridges. Different solvent mixtures were assayed such as: 1 ml water, 1 ml water/MeCN (1:1), 1 ml MeOH, 1 ml MeCN/BO_2^- (0.5 *M*, pH 10.2) (9:1), and 2 ml MeCN/BO_2^- (0.5 *M*, pH 10.2) (8:2), while the best clean-up was obtained with 1 ml MeOH and 1 ml MeCN/BO_2^- (0.5 *M*, pH 10.2) (9:1). The amines retained on the cartridges were eluted with 1 ml of acetic acid solution (0.1 *M*). However, by using this procedure, the recoveries achieved for the different amines assayed are in the range of 40–50%, which means that the sensitivity of the procedure is reduced by up to a factor of two.

3.2.2. Formation, retention and elution on C_{18} cartridges

In order to increase the selectivity and the stability of the derivatives, the reaction products formed in solution were retained into C_{18} cartridges as described in the previous section. To remove the endogenous interferences, a clean-up step was included. The interferences can be eliminated by cleaning the sample with 3 ml of acetonitrile before elution of the reaction products. These results were confirmed by injecting these extracts into a chromatographic system, it being possible to isolate the peak corresponding to the spermine.

Different mixtures (MeOH:water:buffer) were assayed to elute the reaction products. Analogous to the standard solutions, the best results were obtained by eluting with 3 ml of MeOH–borate buffer (9:1).

The kinetic behavior of the reaction products in urine samples was studied. Similar results were obtained for the standards and for the fortified urine samples by applying procedure II. However, the degradation rate constant ($k = 34 \pm 2 \times 10^{-3}$ min^{-1}) calculated from the slope of the regression line (ln Abs vs. time), was higher than that obtained for standard solutions and confirms that the dependence is pseudo-first order. No differences were observed in the rate constant with the urine volume assayed.

The sensitivity obtained by measuring 20 s after the elution of the reaction product was 0.0485 cm mg^{-1} l^{-1}, similar to that obtained for the standard solutions. In these experiments the urine volume employed ranged between 0.05 and 0.1 ml, the sensibility decreasing when higher urine volumes are used. This is mainly due to reagent consumption by the urine endogenous compounds.

In order to increase the volume of urine processed, extraction of the analyte from the urine matrix by a clean-up step using an SPE cartridge as previously described was performed. However, by using this procedure, the recoveries achieved for the different amines assayed are in the range of 40–50% as in the solution derivatization, which means that the sensitivity of the procedure is reduced by up to a factor of two.

3.2.3. On-column derivatization

The sensitivity obtained when the derivatization was performed on column was higher than that obtained by using the other procedures described above. However, direct injection of urine into the column did not allow the identification of the analyte peak due to the high amount of endogenous compounds as can be seen in Fig. 6. This figure also shows no differences between the fortified urine and urine alone, so no total formation of spermide derivative is shown in this case, due probably to the reagent consumption by the endogenous compounds.

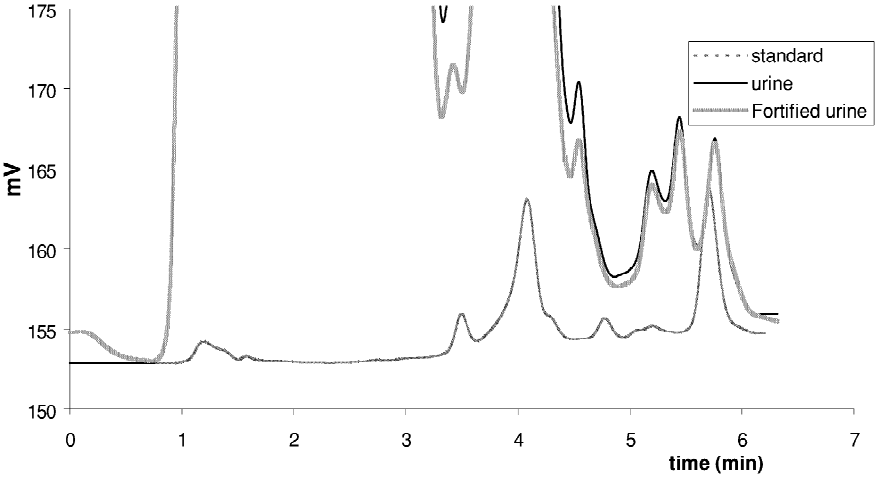

Fig. 6. Chromatograms corresponding to urine sample (blank or fortified) by using on-line derivatization (for more details see Experimental section: procedure III).

On basis of these results, we consider that direct injection is not possible and further research needs to be done in order to optimise the procedure.

3.2.4. Derivatization on packing supports

Similar kinetic behavior is observed for standard and for fortified urine samples. By using this procedure the stability of the reaction product formed into the column increases, and it is stable until 7 min after it is eluted from the column. Other advantages of this approach are that higher urine volumes can be processed (2 ml) directly and it can be adapted to an automatic system [13]. The sensitivity of this procedure (Table 3) is less than the others tested for standards. However, with urine sample, this procedure provides similar results to those obtained with solution derivatization and retention of the formed derivatives, which requires a previous clean-up — acidic elution — and solution derivatization.

3.3. Usefulness of the different approaches

In Table 5 are summarised the most relevant analytical characteristics of the different procedures assayed. Solution derivatization is a sensitive and quick procedure, however the high degradation of the products, especially the spermine, made this procedure less adequate in terms of accuracy and precision. Moreover, solution derivatization usually requires additional steps in order to avoid reagent excess or matrix interferences An alternative in such a case can be the selective retention of the reaction

Table 5
Summary of the most relevant characteristics of the different procedures assayed

Analytical property	Procedure I	Procedure II	Procedure III	Procedure IV
Sensitivity	****	***	****	**
Selectivity	**	***	*	****
Repeatability	**	*	****	***
Reproducibility	**	*	****	***
Stability	*	**	****	***
Time analysis	***	*	****	**
Cost	****	***	*	**

The higher the number of asterisks, the better the characteristic. Procedure I: solution derivatization. Procedure II: solution derivatization followed by retention on solid supports. Procedure III: on-column HPLC derivatization (on-line). Procedure IV: derivatization on solid supports (off-line).

products on the solid supports, which allows similar sensitivities and higher stabilities than those obtained by solution derivatization. Although this automatization has been performed by Saito et al. [8], no application to real samples has been carried out.

The reaction products formed on the solid support (C_{18}) presented higher stability, and higher selectivity, and although the sensitivity obtained was lower it can be improved. This procedure can be adapted to an HPLC system, and the derivatization can be performed on line by a previous retention of the analyte on a solid support placed before the analytical column [8,14].

On-line derivatization in an HPLC system shows higher sensibility, selectivity and stability of the reaction products formed with standard solutions. However, it cannot be applied to the direct analysis of urine samples, which is according to the results described in the literature [9].

From the point of view of economics, solution derivatization can be considered the cheapest procedure, however it presents many disadvantages, such its poor applicability to real samples. The most expensive is the HPLC procedure. The use of solid supports is rather cheap due to the fact that the same support can be used many times (~100 times).

Taking into account all these considerations, it seems that retention or derivatization on solid supports could be an alternative to the solution derivatization of OPA-derivatives, improving stability, selectivity, minimising the cost of the analysis and broadening applicability to real samples.

4. Conclusions

In this paper, different derivatization procedures of polyamines using OPA as derivatizing reagent, have been optimized. The study has focused on the stability of the reaction products, and sensitivity and versatility of the procedure. The optimized conditions, the analytical properties, and the advantages and disadvantages of all of them are given. The behavior and recoveries of these derivatives in urine samples were similar to those obtained for the standard solutions. On the basis of these experiments, the proposed derivatization using C_{18} cartridges has demonstrated advantages over the other procedures assayed, i.e. increases in the stability of the OPA-derivative, and could be also suitable for routine analysis of polyamines (spermine) in urine samples.

Acknowledgements

The authors are grateful to the DGICYT for financial support received for the realisation of Project PB97-1387.

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