Urine polyamines determination using dansyl chloride derivatization in solid-phase extraction cartridges and HPLC

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The derivatization of biogenic amines such as putrescine, cadaverine, spermidine and spermine with dansyl chloride in solid phase extraction cartridges is described. Different types of filling materials were tested in order to have the highest retention of the different analytes. The best results were obtained by using C18 cartridges. The optimal conditions were: amine solution buffered at pH 12, 2 mM dansyl chloride (acetone–bicarbonate solution 20 mM (pH 9–9.5), 2 + 3 v/v) as reagent concentration, room temperature and 30 min reaction time. The developed procedure was applied to the determination of these polyamines in urine samples from healthy controls and cancer patients using HPLC with 1,7-diaminoheptane as internal standard. The concentrations ranged from 0.5 to 5 μg mL^{-1} and the detection limits were 10 ng mL^{-1} for all polyamines. By concentrating the urine extracts, the detection limits were improved down to 2 ng mL^{-1}. The accuracy and the precision of the method were tested. The proposed dansylation method is advantageous with respect to solution dansylation. It improves the total analysis time, avoids high temperatures that can affect the thermal stability of the derivatives and could make possible the automation of the procedure.

Introduction

Polyamines (such as putrescine, cadaverine, spermine or spermidine) are essential for normal growth and cellular differentiation.[1–3] They are commonly present in significant amounts both in prokaryotic and eukaryotic cells. Malignant cell proliferation is associated with increased cellular polyamine metabolism.[4] Several diagnostic assays based on polyamine detection have been developed to screen for cancer, to evaluate efficacy of therapy and to detect relapse.[5] Researchers have demonstrated that tumour cells contain a much higher concentration of polyamines, and patients with many types of cancers have probably an enhanced urinary excretion of polyamines.

Several methods have been described for their determination in biological samples, high performance liquid chromatography (HPLC) being the one preferred. The direct detection of polyamines is difficult because they do not absorb in the ultraviolet region and, consequently, they do not have native fluorescence, required in most of these procedures of derivatization. However, most of them require an extraction procedure to remove interfering derivatives or reagent excess. These features prevent automatization of the derivatization procedure and decrease reproducibility. Pre-column or post-column derivatization, followed by spectrophotometric and fluorimetric detection, are, therefore, commonly applied. Fluorimetric reagents such as dansyl chloride,[6] fluorescamine,[7] *o*-phthaldialdehyde(OPA)–2-mercaptoethanol[8,9] or *o*-phthaldialdehyde–ethanethiol[10] have been proposed for determination of polyamines. A number of pre-column derivatization techniques have been developed for HPLC with UV/VIS spectrophotometric detection.[11] They include reactions with benzoyl chloride,[12] *p*-toluenesulfonic chloride (tosyl chloride), 2,4-dinitrofluorobenzene, 4-fluoro-3-nitrobenzotrifluoride, quinoline-8-sulfonyl chloride and 4-dimethylaminoazobenzene-4′-sulfonyl chloride (dansyl chloride).

The detection limits reached with the fluorimetric reagents are generally better than those obtained with the spectrophotometric reagents. The most commonly employed reagents for converting polyamines into fluorescent products are dansyl chloride and OPA–thiol. The stability of the dansyl derivatives is better than that achieved with the isoindoles formed with the OPA–thiol reagents. One advantage of using dansyl derivatives is the possibility of forming chemiluminiscence with oxalic acid bis(2,4,6-trichlorophenyl ester)–H_2O_2, which can increase sensitivity and selectivity.

During the last five years, this research group has been studying derivatization procedures off- or on-line on solid phase supports. 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 and concentrate the analytes and, next, the derivatization agent is passed through and the derivatized analytes are retained. Some applications, based on this methodology, have been described for amphetamine and related compound determination in urine samples, by using different reagents such 9-fluorenylmethyl chloroformate (FMOC), OPA, 1,2-naphthoquinone 4-sulfonate (NQS)[13–15] or dansyl chloride (Dns-Cl).[16]

This paper extends our methodology to the analysis of biogenic polyamines. A simple and rather quick off-line HPLC procedure for both clean up and derivatization of polyamines on solid phase extraction is proposed. The optimal conditions have been established and the final procedure has been applied to the determination of these amines in urine samples.

Since the determination of polyamines is of great interest in cancer urinary profiles (due to the concentration levels being not completely clear) or in metabolic studies, the potential of the described approach has been tested by analysing these biogenic amines in the urine of healthy volunteers or cancer patients.

Experimetal

Apparatus

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

loop injector of 100 μL, and a high-pressure six-port valve (Rheodyne model 7000; Cotati, CA, USA). A fluorescence detector (Hewlett-Packard, 1050 Series) (flow cell, 5 μL) was coupled in series and linked to a data system (Hewlett-Packard HPLC Chem Station) which was used for data acquisition and storage. The chromatographic signal was excitation at 252 nm and emission at 500 nm for fluorescence. A Vac Master –10 sample processing station (International Sorbent Technology, Hengoed, UK) at a flow-rate of about 5 mL min^{-1} was used. A rotary evaporator was also used. All the assays were carried out at room temperature.

Reagents

All the reagents were of analytical grade. Acetonitrile, methanol and acetone (Scharlau, Barcelona, Spain) were of HPLC grade. Putrescine dihydrochloride, cadaverine dihydrochloride, spermine tetrahydrochloride, spermidine trihydrochloride, dansyl chloride and 1,7-diaminoheptane were obtained from Sigma (St. Louis, MO, USA). Sodium hydrogencarbonate (Probus, Badalona, Spain), sodium hydroxide (Panreac, Barcelona, Spain), imidazol (99%) from Sigma and nitric acid was also used.

Columns and mobile phases

Bond Elut C18 200 mg and Bond Elut Certify 130 mg from Varian (Harbor City, CA, USA), high performance extraction disk cartridges C18 from 3M Empore (St. Paul, MN, USA) and LiChrolut EN (200 mg) and LiChrolut TSC (300 mg) from Merck (Darmstadt, Germany) were used to retain the analytes and later to perform an off-line derivatization.

A C18 Lichrospher (125 × 4 mm id, 5 μm particle size) (Merck), column was used as an analytical column for separation of the amine derivatives. An acetonitrile–imidazol solution (1 mM, pH 7.0) (70 + 30 v/v) mixture in gradient elution mode was used as the eluent at a flow rate of 1 mL min^{-1}. The gradient used was 70% of acetonitrile at zero time, 90% at 5 min, and 70% at 9 min. After 9 min the percentage of acetonitrile was kept constant. All the solvents were filtered through a 0.45 μm nylon membrane (Teknokroma, Barcelona, Spain) and degassed with helium before use.

Preparation of solutions

Standard solutions of 2.0 g L^{-1} Dns-Cl were prepared by dissolving the pure compound in acetone. Standard solutions of the amine compounds were prepared by dissolving the pure compounds in water (1000 μg mL^{-1}). Working amine solutions were prepared by diluting the standard solutions in water. The pH was adjusted by adding the corresponding amount of hydrogencarbonate buffer which was prepared by dissolving in water the appropiate amount of sodium hydrogen carbonate and then adjusting the pH with 10% NaOH (w/v). All solutions were stored in the dark at 4 ºC.

Solution derivatization

The amines were derivatized according to the method described by Marcé *et al.*[17] To 0.1 mL of amine, 1 mL of 10 mM hydrogen carbonate buffer (pH 9.0) and 0.9 mL of acetone containing 1.0 mM dansyl chloride were added successively, and the mixture was incubated for 10 min at 70 ºC. An aliquot (20 μL) of the solution was injected into the HPLC system.

Fractions I and II obtained in the pH experiment were derivatized by following this procedure. Fraction I corresponds to the fraction collected when the amine solution is passed through the column and fraction II is collected when the reagent solution is passed through the column.

Derivatization on solid-phase supports

Solid-phase extraction cartridges were conditioned by drawing 1.0 mL of methanol through followed by 1.0 mL of hydrogen carbonate buffer (pH 12). Aliquots (1 mL) of the samples were then transferred to the cartridges and 0.5 mL of Dns-Cl reagent prepared in acetone–hydrogencarbonate buffer solution (20 mM, pH 9.5), (2 + 3 v/v) was flushed through the cartridges. After a given reaction time, the cartridges were dried under vacuum with air at a flow of 5 mL min^{-1}. The derivatives formed were desorbed from the cartridges with 1 mL of acetonitrile. A 20 μL aliquot of the resulting mixture was finally injected into the chromatographic system.

Urine samples

Untreated urine samples were spiked with the analytes (putrescine, cadaverine, spermidine, and spermine) at a concentration range of 0.5 and 5 μg mL^{-1}. 1,7-Diaminoheptane was included in the sample as internal standard (IS) at a concentration level of ca 2.5 μg mL^{-1}. These samples were alkalised with NaOH and carbonate buffer to pH 12. Volumes of 1 mL of these samples were placed into conditioned C18 cartridges. Then the analytes were derivatized in the solid-phase extraction cartridges as described above. The percentage of analyte recovered after clean-up plus derivatization was calculated by comparing the peak area obtained for a particular assay with those obtained for standard solutions containing an equivalent amount of analyte. Each sample was assayed in triplicate.

Real samples

Five urine samples from cancer patients and ten corresponding to healthy volunteers were analysed. A portion (1 mL) of untreated urine sample was placed in the column together with the IS and the alkalised medium. Then, the analytes were derivatized as described above.

Concentration procedure: 1 mL of the organic phase (acetonitrile) was removed and dried under a N_2 stream while the tube was standing at 40 ºC in a water bath. The residue was dissolved in 250 μL of acetonitrile. Finally, a 20 μL sample was injected onto the HPLC system.

Two of the samples from healthy volunteers were also processed by a conventional procedure:[18] to 2.0 mL of urine in a test-tube, 0.2 mL of sodium hydroxide solution (10% in water) and 2.0 mL of diethyl ether were added successively. The tube was capped and shaken vigorously for 2 min. After centrifuging at 1000*g* for 10 min, the ether layer was collected. The extraction procedure was repeated twice. The ether extract was dried in a rotary evaporator system after the addition of a drop of diethyl ether containing 0.1 M HCl. The derivatization procedure was performed according to the description given above for solution derivatization.

Results and discussion

In order to have a reference for the derivatization reaction, polyamines were derivatizated in solution according to the conditions described by Marcé *et al.*[17] (Fig. 1, Procedure I). The steps related to the sample clean-up and removal of the reagent excess were avoided (Fig. 1, Procedure II). No differences were found between both procedures and no interference of reagent

was obtained at the retention time of the analytes. These results were taken as 100% of derivatized product.

Dansylation on solid-phase supports

The retention conditions of the amines on the solid support and their reaction conditions with dansyl chloride were studied.

C18 cartridges were initially used to retain the analytes and to perform the derivatization procedure. Amine solutions at different pH values ranging from 10 to 13 were tested. Three different fractions were analysed. Fraction I corresponded to the portion collected when the amine solution was passed through the column. Fraction II was collected when the reagent solution was passed through the column and fraction III was the elution of the reaction products formed in the cartridge. In order to show the retention of the biogenic amines or the loss of those species when the reagent was flushed, fractions I and II, respectively, were derivatized according to the dansylation in solution.

1,7-Diaminoheptane, spermine and spermidine did not give analytical signals in fractions I and II. Thus, those polyamines were totally retained in the cartridge. Fig. 2 shows the percentage of the total analytical signal obtained in the different fractions for cadaverine and putrescine. The higher the solution pH the lower was the amount of analyte eluted in fractions I and II because the retention of both analytes in the cartridge increased with the pH. Then, the formation of the derivatives in the cartridge was higher as the solution pH was also higher (see fraction III of Fig. 2) The results at pH 13 are not shown in the figure because in these conditions the reagent was destroyed and no analytical response was obtained.

The analytical signals obtained for the derivatives formed in the cartridge (fraction III) for all polyamines as a function of the pH of the solution flushed through, are given in Fig. 3. We selected amine solutions buffered at pH 12 from Fig. 3 in order to improve spermine determination, which is the most retained species and the last eluted in reversed liquid chromatography.

To establish the optimal pH for polyamine retention, the reaction conditions in the column were investigated, and parameters such as pH, reagent concentration and time were studied.

The reaction was carried out at a basic pH, and the influence of this parameter was studied in the range 8–10. No big differences were observed by modifying the pH of the flushed solution in this range, so, in accordance with our paper[16] concerning dansylation in C18 supports of amphetamines, a reagent mixture of hydrogen carbonate solution at pH 9.5 and Dns-Cl in water–acetone (3 + 2 v/v) was used.

The effect of the Dns-Cl concentration was evaluated in the range from 1.85×10^{-4} to 1.85×10^{-3} M. As can be seen in Fig. 4 the analytical signal increased with the reagent concentration. Higher amounts of reagent could not be used in order to avoid analyte elution and the formation of undesirable reagent compounds.

Three different reaction times were studied (5, 15 and 30 min). Analytes such as putrescine, cadaverine or 1,7-diaminoheptane (SI) did not show any change with time, however, as the response of spermine and spermidine increased with time, 30 min was chosen as the most suitable reaction time.

The results obtained using the selected conditions were compared with those obtained by performing solution derivatization after working the solid-phase extraction procedure without dansyl chloride. The efficiency of the derivatization step in the cartridge was tested. As for putrescine, cadaverine and 1,7-diaminoheptane, similar amounts of reaction products

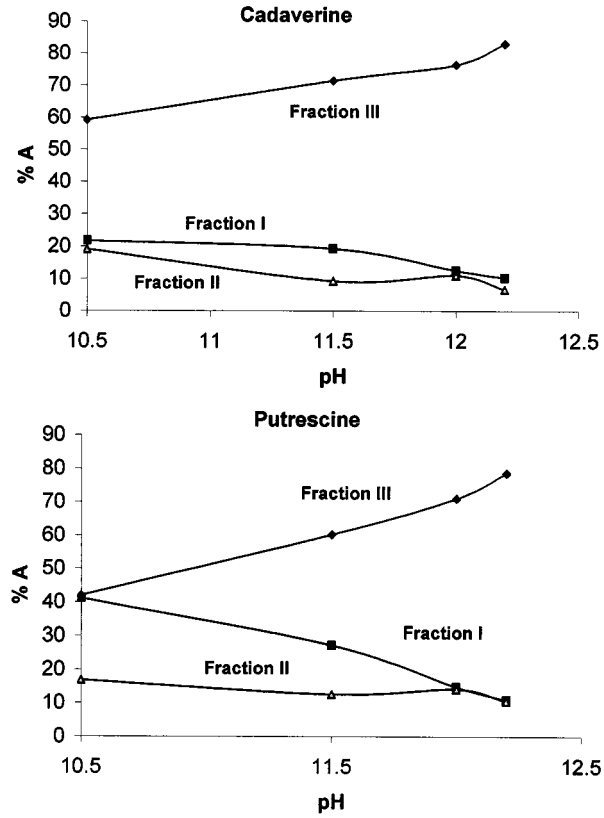

Fig. 2 Effect of the pH of the polyamine solution flushed through the cartridge. Reaction conditions: room temperature (25 °C), sodium hydrogen carbonate solution (20 mM) pH 9.5, putrescine 1.1 μg mL^{-1} and cadaverine 1.20 μg mL^{-1}. For the meaning of I, II and III fractions see text.

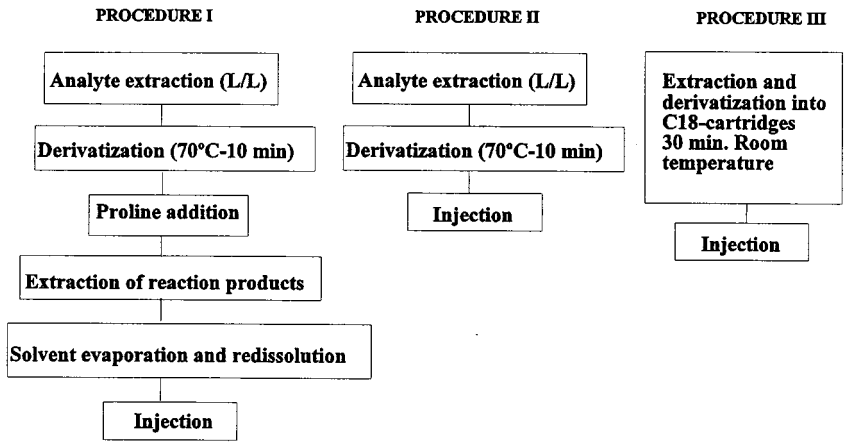

Fig. 1 Different derivatization procedures. I, solution derivatization according to Marcé *et al.*,[17] II, solution derivatization procedure modified; III, derivatization in solid cartridges.

were obtained, being nearly 100%. However, for spermine and spermidine the amounts of reaction products were *ca.* 60–70%.

In order to see whether the reaction between the amines and Dns-Cl was dependent on the solid support, different materials were tested. In Fig. 5 the recoveries with respect to dansylation in solution for each analyte by using different types of cartridges and performing the derivatization at optimal conditions are given. The best results of retention and derivatization were obtained by using the C18 packing, and it was the cartridge selected.

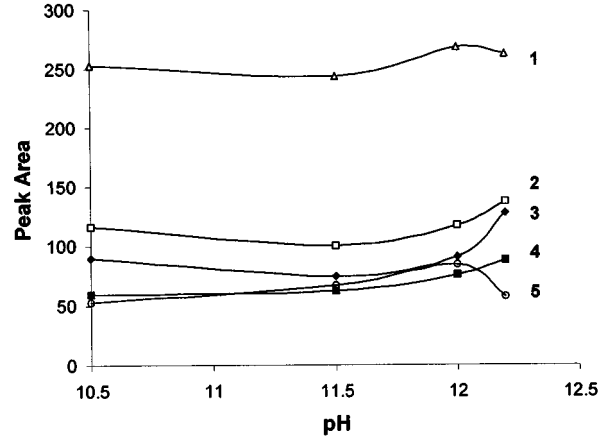

Fig. 3 Effect of the pH of the polyamine solution flushed through the cartridge. Reaction conditions: room temperature (25 °C), sodium hydrogen carbonate solution (20 mM) pH 9.5, dansyl chloride 2 mM. 1,7-Diaminoheptane 2.26 μg mL^{-1} (1), cadaverine 1.20 μg mL^{-1} (2), putrescine 1.10 μg mL^{-1} (3), spermidine 1.19 μg mL^{-1} (4) and spermine 1.25 μg mL^{-1} (5).

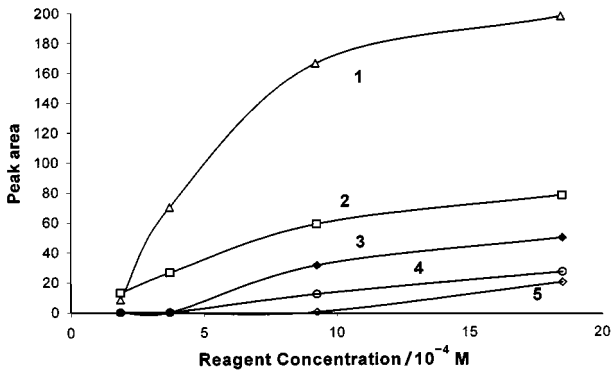

Fig. 4 Effect of the reagent concentration (Dns-Cl) on the reaction rate. Conditions: room temperature (25 ºC), sodium hydrogen carbonate solution (20 mM) pH 9–9.5. 1,7-Diaminoheptane 3 μg mL^{-1} (1), cadaverine 1.49 μg mL^{-1} (2), putrescine 1.18 μg mL^{-1} (3), spermidine 0.95 μg mL^{-1} (4), and spermine 0.47 μg mL^{-1} (5). Dns-Cl concentration was 0.1–15 mM.

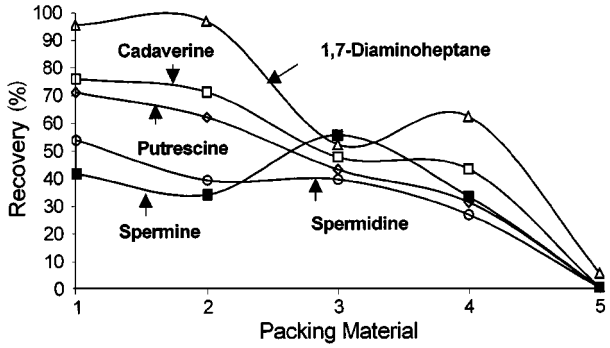

Fig. 5 Solid-phase extraction dansylation percentage recovery with respect to solution dansylation *vs.* solid support: 1, C18; 2, TSC; 3, Certify; 4, Disk; and 5, EN. Conditions: putrescine 1.1 μg mL^{-1}, cadaverine 1.20 μg mL^{-1}, spermidine 1.19 μg mL^{-1}, spermine 1.25 μg mL^{-1} and 1,7-diaminoheptane 2.26 μg mL^{-1}. For experimental details see Experimental.

Analytical properties

According to Won Suh *et al.*[19] for quality control of urine samples, the concentration range chosen was 500 ng mL^{-1} up to 5 μg mL^{-1}. We selected urine with the lowest amounts of biogenic amines as a control in order to study the calibration graph in urine and the accuracy of the method. Fig. 6 shows the chromatograms corresponding to unspiked and spiked urine sample, respectively. The equations of the calibration graphs were: $y = -0.0113 + 0.6258C$ ($r = 0.999$) for putrescine; $y = 0.0239 + 0.7438C$ ($r = 0.999$) for cadaverine; $y = 0.0031 + 0.4025C$ ($r = 0.998$) for spermidine and $y = -0.0189 + 0.2722$ C ($r = 0.999$) for spermine. The linearity was good in this range.

The limit of detection (LOD) was calculated as the amount of analyte giving a peak height three times the maximum noise peak height of a blank biological sample observed at the retention time of each analyte. The LOD was 10 ng mL^{-1}.

The recoveries obtained when spiked urine samples were processed are shown in Table 1. In all cases, 1,7-diaminoheptane was used as IS. This table also gives the repeatability and reproducibility achieved between days.

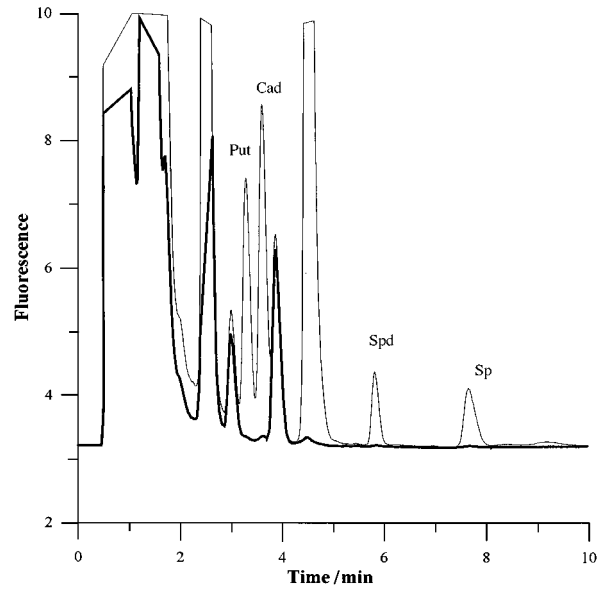

Fig. 6 Chromatograms obtained for blank urine (–) and spiked with polyamines with putrescine (Put) 0.83 μg mL^{-1}, cadaverine (Cad) 0.88 μg mL^{-1}, spermidine (Spd) 0.88 μg mL^{-1} and spermine (Sp) 0.93 μg mL^{-1}, (–). For experimental details see Experimental.

Table 1 Analytical data for the determination of free polyamines in urine samples. Recoveries take into account all the concentrations tested

Analyte	Recovery (%) (n = 8)[a]	Reproducibility between day (%) (n = 3)	Repeatability precision (%) (n = 8)
Putrescine	95 ± 4	3	4
Cadaverine	90 ± 8	6	8
Spermidine	80 ± 7	3	7
Spermine	82 ± 6	6	6

[a] Recoveries considering the calibration graph with standards. 1,7-Diaminoheptane was used as internal standard.

Table 2 gives the accuracy obtained for the urine samples spiked with different concentrations. As can be seen in this table the accuracy is good independently of the polyamine concentration.

Determination of polyamines in urine samples

We analyzed ten different urine samples of healthy subjects and we found that the biogenic amine concentrations were near to the detection or determination limits. We also analyzed two of these samples by using liquid–liquid extraction as sample clean up and solution dansylation and similar results were obtained.

In order to improve the detection limits of the method, a concentration procedure was proposed. The elution volume (containing the reaction products) was removed and dried under a N_2 stream while the tube was standing at 40 ºC in a water bath. The proposed procedure improved the detection limits to 2 ng mL^{-1}. Fig. 7 shows the chromatograms of a healthy volunteer (sample 6) and the five urine samples (samples 1 to 5) from cancer patients. As can be seen variation in the shape of the chromatogram is observed.

The putrescine concentration found was 0.33, 0.44, 0.51 and 0.69 μg mL^{-1} for urine samples 1, 2, 4 and 5, respectively. The cadaverine concentration was 0.26, 0.36, 0.18 and 0.4 μg mL^{-1} for samples 1, 2, 4 and 6, respectively. The spermidine concentration was 0.20, 0.08, 0.13, 0.05, 0.03 and 0.18 μg mL^{-1} for samples 1, 2, 3, 4, 5 and 6, respectively and the spermine concentration was 0.12, 0.03, 0.03 and 0.12 μg mL^{-1} for samples 1, 2, 4 and 6, respectively. All analytes were found in urine samples 1, 2 and 4. However in urine sample 3 only spermidine was found. Spermine was found in the six urine samples. Won Suh *et al.*[19] found that the ratio putrescine/spermidine in urine was significantly greater in cancer patients than in normal subjects. This significant difference of ratio values supports the well known fact that extracellular putrescine concentrations may reflect either rapid tumor growth or tumor cell loss.[20] Although a putrescine/spermidine ratio cannot be calculated for the healthy person because putrescine is below the detection limit, the results obtained could be in agreement with those of Won Suh *et al.*[19] According to other authors[19,21,22] greater differences can be expected in the acethylated form and can correspond to other differences observed in the chromatograms. More work is needed in order to determine definite profiles of these biogenic amines in cancer patients and healthy volunteers. The proposed method can serve in this sense as it is demonstrated.

Table 2 The accuracy in the determination of free polyamines in spiked urine samples.

Analyte	Added conc./ μg mL^{-1}	Found conc./ μg mL^{-1} (n = 3)	Error (%)
Putrescine	0.83	0.79 ± 0.12	+4.89
	1.65	1.74 ± 0.06	−5.43
	2.20	2.17 ± 0.27	+1.39
	3.30	3.26 ± 0.30	+1.15
Cadaverine	0.88	0.83 ± 0.66	+5.35
	1.77	1.85 ± 0.13	−4.69
	2.35	2.33 ± 0.14	+0.99
	3.53	3.53 ± 0.21	−0.09
Spermidine	0.88	0.90 ± 0.06	−2.91
	1.76	1.73 ± 0.00	+1.21
	2.34	2.26 ± 0.11	+3.50
	3.51	3.65 ± 0.06	-3.90
Spermine	0.93	0.85 ± 0.03	+8.31
	1.85	1.96 ± 0.00	-5.90
	2.47	2.46 ± 0.08	+0.28
	3.7	3.69 ± 0.24	0.32

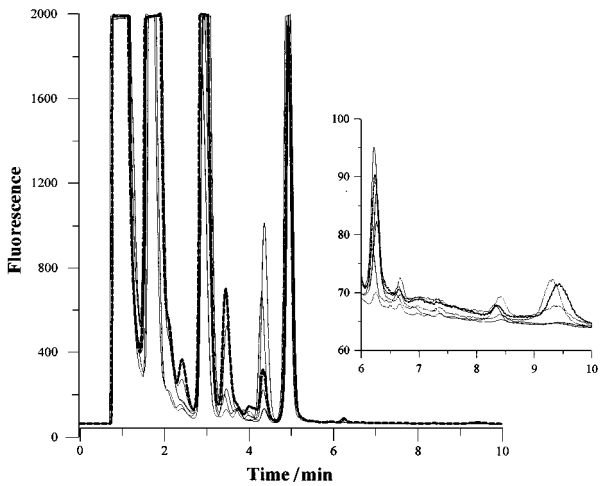

Fig. 7 Chromatograms corresponding to concentrated urine from a healthy volunteer (--) and urine from cancer patients (-). For experimental details see Experimental.

Conclusions

This paper shows the possibility of using Dns-Cl reagent to perform polyamine derivatization in urine samples. The derivatization is performed in C18 solid phase cartridges, which permits the clean-up and derivatization of the analytes on the solid support, decreasing sample handing and time of analysis. The time of analysis is less than 1 h, in which 30 min corresponds to the derivatization reaction in the cartridges; during this time other work can be done. However, the conventional procedure takes more than 1.5 h working all the time using tedious operations (double liquid–liquid extraction, evaporation of the solvent).

The proposed procedure also avoids heating the mixture and allows the use of more polar solvents such as acetonitrile than those employed in liquid–liquid extraction. The volume of solvent employed is smaller than that required in the conventional method.

The procedure has been applied to spiked and real urine samples from healthy volunteers and cancer patients, respectively. The results obtained from spiked urine samples are accurate and precise, which allows validation of the procedure. The detection limits have been improved to 2 ng mL^{-1} by concentration of the urine extracts.

By using this procedure, the determination of polyamines (putrescine, cadaverine, spermine and spermidine) in urine of unknown cancer patient samples can be performed with satisfactory sensitivity, reproducibility and a minimum time.

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