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POLYMERIC BENZOTRIAZOLE REAGENT FOR THE OFF-LINE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DERIVATIZATION OF POLYAMINES AND RELATED NUCLEOPHILES IN BIOLOGICAL FLUIDS*

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SUMMARY

A polymeric benzotriazole reagent containing a 9-fluorenylmethyleneoxycarbonyl (FMOC) group has been synthesized, characterized, and its derivatizations, off-line, for three polyamines, have been optimized with regard to solvent, time, and temperature. An authentic FMOC derivative of cadaverine has been prepared, characterized, and used as the external standard for quantitation of off-line derivatizations and identification of final derivatives. Actual percent derivatizations have been determined, rather than just percent disappearance of starting material. The polyamines in urine or other biological fluids can be derivatized without organic solvent or solid phase extraction, but rather *in situ* by the simple addition of the polymeric reagent to the fluid, incubation for a few minutes at room or elevated temperature, filtration and direct injection. Derivatizations could also be performed by transferring a small volume of the hydrolyzed and filtered biological fluid to a disposable pipette containing the polymeric reagent. Derivatization was then followed by elution, filtration, and direct injection onto the high-performance liquid chromatographic (HPLC) system. Automation of the overall polymeric derivatization, filtration, HPLC injection, separation, detection, quantitation, and data acquisition-interpretation is suggested.

The polymeric reagent has been utilized for the qualitative and quantitative determination of cadaverine and putrescine, normally occurring polyamines, in human urine. These levels were compared with the corresponding literature values for healthy human subjects, and the values were found to be in excellent agreement. This novel derivatization approach, though off-line, provides for a much simpler, more rapid, and more efficient conversion of these and related polyamines or nucleophiles

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to derivatives having vastly improved chromatographic detection properties in HPLC. The final derivatives contain the FMOC group, making them extremely chromophoric and fluorophoric, and providing trace detection at ppb (μg/l) and sub-ppb levels. The overall approach is recommended for these and other biologically occurring polyamines, in fluids and tissues, as well as related bioorganic and biologically active nucleophiles, including drugs and their metabolites.

INTRODUCTION

In recent years, we and others have described certain novel polymeric or solid phase reagents that have proven advantageous in both off-line and on-line derivatizations related to final high-performance liquid chromatographic (HPLC) determinations[1–15]. We have most recently described the synthesis, characterization, optimization of derivatizations, percent derivatizations, and analytical figures of merit for a particular polymeric benzotriazole reagent containing the 9-fluorenylmethylene oxycarbonyl (FMOC) tag[15]. The derivatization of standard nucleophiles using this polymeric benzotriazole reagent provided additional selectivity, and also decreased detection limits for primary and secondary amines or polyamines by both ultraviolet (UV) and fluorescence detection in HPLC. However, no real world applications for this polymeric reagent were described. The label or tag was designed to be detector versatile, so that the derivative(s) would be amenable to several different detection modes, in this case UV–fluorescence[15]. Gao *et al.*[15] have done a complete study using a related polymeric benzotriazole *o*-acetylsalicyl reagent for derivatizations of simple amines. These particular derivatives were totally analogous to others described by us for a related polymeric anhydride reagent containing the same tag[13].

When one performs an analysis involving a derivatization, solution or solid phase, the derivative must often be separated from excess derivatizing reagent and any other species present in the sample matrix. Of course, in the case of solid phase reagents, excess derivatizing reagent is not present in the final derivative solution, though sample components still are[1,2]. The separation of analyte derivative from potential interferents may be achieved through chromatography, but if several detection modes are available, separation of the derivative may be more easily accomplished through selective detection. This was singularly exemplified in the recent reports by Colgan *et al.*[11,12,14].

Polyamines, such as putrescine and cadaverine, were chosen as analytes due to their physiological and biochemical significance. The concentrations of polyamines in the urine of cancer patients and in the plasma of uremic patients are elevated[16–18]. Accurate and precise quantitation for polyamines in physiological fluids have been of considerable importance and interest. HPLC coupled with fluorescence detection offers high sensitivity and good specificity as a general approach for polyamine assays at trace levels, following appropriate derivatization. Various solution derivatizing reagents, such as: *o*-phthalaldehyde (OPA), fluorescamine (Fluram), and 5-dimethylaminonaphthalene-1-sulphonyl (Dansyl) chloride have been used for polyamine derivatizations in the past[19–26].

All of these solution reagents and reactions lead to polyamine derivatives with strong fluorescent properties and excellent detection limits. However, none of the

existing derivatizations have involved solid phase reagents, such as the polymeric benzotriazole FMOC[15]. This new polymeric reagent has been synthesized using standard procedures, based on a polystyrene–divinylbenzene support, commercially available. The FMOC tagging moiety was purposely incorporated into the final polymeric reagent, which resulted in fluorenylamino type derivatives, actually carbamates.

This reagent has been used in an off-line, pre-column mode, prior to HPLC injection, for a number of polyamines. Its use in the on-line, pre-column mode, has been attempted, but found unsuccessful in a repetitive manner[27]. A standard FMOC derivative of cadaverine was prepared, characterized by physical and spectral properties, as well as elemental analysis, and used as an external standard to determine percent derivatizations with the polymeric reagent[15]. The reagent itself was characterized by its method of synthesis and loading determinations. The feasibility and applicability of this reagent for the derivatization of polyamines, such as cadaverine and putrescine, in biological fluids has now been examined under optimized conditions. This approach can provide significant advantages over all related solution type derivatizations with the same and related nucleophiles. The approach may be applicable for routine clinical and biomedical–biochemical analyses in most, if not all, aqueous, organic, or aqueous–organic sample matrices.

The limits of detection (LODs) with fluorescence are 1–2 pmol for typical polyamines using the above approach, which is comparable to or better than all existing solution derivatization methods[19–26]. The linear calibration range is 3–4 orders of magnitude concentration of each polyamine.

EXPERIMENTAL

Chemicals, reagents and solvents

Macroporous polystyrene–divinylbenzene copolymer (particle size, d_p 60–90 μm, Porapak Q) was obtained from Waters Chromatography Division (Millipore, Milford, MA, U.S.A.). Chemicals used were obtained from a variety of commercial sources, including: Aldrich (Milwaukee, WI, U.S.A.), Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), J. T. Baker (Phillipsburg, NJ, U.S.A.), Alfa Products (Danvers, MA, U.S.A.), Sigma (St. Louis, MO, U.S.A.). These chemicals were of the highest purity available and were used without further purification. HPLC solvents were obtained from EM Science (Cherry Hill, NJ, U.S.A.), as their Omnisolv HPLC grade. All HPLC solvents were used after filtration through a 0.45-μm solvent filter (GVWP, Millipore, Bedford, MA, U.S.A.) and degassed under vacuum with stirring.

Apparatus

The HPLC system consisted of a Waters Model 6000A solvent delivery system, a Waters Model U6K syringe loading injection valve, and a Brown Boveri Model SE 120 dual-pen recorder (Brown Boveri, Metrawatt/Goerz Division, Vienna, Austria). Chromatographic columns consisted of a Waters μBondapak™ C_{18} reversed-phase, 30 cm × 7.8 mm I.D. (semipreparative), a Waters μBondapak C_{18} reversed-phase, 30 cm × 3.9 mm I.D., or an 8 mm I.D. Radial-Pak Resolve™ C_{18} column used in a Model RCM-100 radial compression module. The detectors consisted of a Waters Model 480 variable-wavelength UV detector and a Hitachi Model F1000 fluorescence

spectrophotometer. At times, a Hewlett-Packard Model 3380A reporting integrator (Hewlett Packard, Palo Alto, CA, U.S.A.) was used.

The instrumentation used to characterize the isolated standards or monitor creatinine levels consisted of a Varian Model XL-300 NMR Spectrometer (Varian, Palo Alto, CA, U.S.A.), a Perkin-Elmer Model 599B infrared spectrophotometer (Perkin-Elmer, Analytical Instruments, Norwalk, CT, U.S.A.), a Thomas Hoover capillary melting point apparatus (Arthur H. Thomas, Philadelphia, PA, U.S.A.), a Perkin-Elmer Model Lambda 3B UV–VIS spectrophotometer equipped with the Perkin-Elmer data station, a Milton Roy Model Spectronic 1201 scanning UV–VIS spectrophotometer [Milton Roy, Analytical Products Division (formerly division of Bausch & Lomb), Rochester, NY, U.S.A.], and a Nuclide magnetic sector mass spectrometer (Nuclide, State College, PA, U.S.A.). At times a Hewlett-Packard Model HP 1040A linear diode array UV–VIS detector was used to confirm the purity of standards.

Preparation and reaction conditions of the polymeric benzotriazole FMOC reagent

The entire synthetic procedure for preparation of the final polymeric benzotriazole FMOC reagent (I) was indicated in our previous paper[15]. Because the method of synthesis and its reactions with standard amines or polyamines have been presented elsewhere in the literature, these are not repeated here. The reader is referred to the literature for additional, specific information[15,27,28].

Characterization of the polymeric reagent

The amount of labelling moiety in one gram of the polymeric support was determined by a base-initiated hydrolysis of the polymeric reagent. This procedure was used to demonstrate if the synthesis was successful. It also indicated how much derivatizing reagent or tag had been incorporated into the final polymeric support. Though we have previously described the method of characterization for the analogous polymeric benzotraizole *o*-acetylsalicyl reagent, the hydrolysis information for the FMOC incorporated reagent I was not described[15].

Hydrolysis reaction of polymeric benzotriazole FMOC reagent. Hydrolysis and quantitation of the released tag or its analogue is an accepted method for the determination of loading of a polymeric tagging/derivatizing reagent. The polymeric benzotriazole FMOC reagent (137 mg) (I) was added to 10 ml dioxane and 20 ml potassium hydroxide solution (0.2 *M*) and refluxed for 4 h. After hydrolysis, the solution was acidified to pH 3, and the reaction product was extracted twice by 50 ml chloroform. The organic phase was collected and the solvent was evaporated under vacuum. The reaction product was redissolved in 20 ml acetonitrile, and 25 μl of this solution was injected into the HPLC system. The same procedure was repeated for a reaction blank, except that there was no potassium hydroxide added. The LC UV chromatogram of the hydrolysis product showed an unknown peak at a retention time of 3.2 min under the following chromatographic conditions: 8 mm I.D. Radial-Pak Resolve C_{18} column used in a RCM-100 module; mobile phase: acetonitrile–water (90:10); flow-rate: 2.0 ml/min; UV at 254 nm.

Preparation, isolation and characterization of dibenzofulvene. Hydrolysis of the polymeric benzotriazole FMOC reagent gave essentially an unknown product which was suspected to be dibenzofulvene (II)[29]. The possible mechanism of this

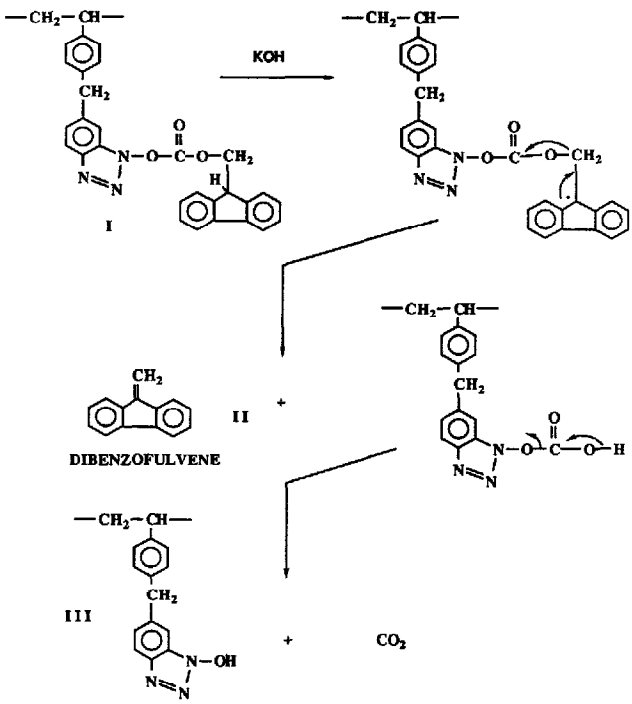

Fig. 1. Possible mechanism of the hydrolysis reaction of polymeric benzotriazole fluorenylmethyl (FMOC) reagent, I, leading to formation of dibenzofulvene, II.

hydrolysis reaction is shown in Fig. 1. To characterize the polymeric benzotriazole FMOC reagent, it was necessary to quantitate this product and thereby determine the loading. Base-catalyzed hydrolysis of 9-fluorenylmethanol could have provided the desired dibenzofulvene, and that could have been used as an external standard. Base hydrolysis of 9-fluorenylmethanol by potassium hydroxide did give one major product. 9-Fluorenylmethanol (1.27 mmol) was dissolved in 15 ml acetonitrile, and this was mixed with 15 ml potassium hydroxide solution (1.27 mmol). The mixture was refluxed for 2 h, and the reaction mixture was extracted with 80 ml chloroform. A white precipitate in the organic layer, which might have been an insoluble polymer of dibenzofulvene, was discarded. The organic solvent was evaporated under vacuum, and the residue was partially dissolved in acetonitrile. The solution was filtered again, and the filtrate was injected into the HPLC system, with conditions as above. Again, a single peak at 3.2 min was observed, strongly suggesting the hydrolysis formation of the same product from the polymeric FMOC reagent (I).

To isolate the desired product, the solution from the above hydrolysis reaction was subjected to semipreparative LC using isopropanol–hexane (3:97) as the mobile phase. Dibenzofulvene (15 mg) was isolated. Since dibenzofulvene was only partially soluble in various solvents, such as acetonitrile, dioxane, chloroform and hexane, and no sharp melting point was observed up to 250°C, we believed most of the dibenzofulvene was polymerized during the collection procedure[29]. The electron impact (EI) mass spectrum of dibenzofulvene showed a base peak at *m/e* 179, which may

have been the molecular ion. Due to the solubility problem in chloroform, the NMR spectrum was not reliable. However, it did show an olefinic methylene group at 6.1 ppm (singlet) and aromatic hydrogens between 7.2–7.8 ppm (multiplet), as demanded by the structure. Because the dibenzofulvene hydrolysis product was not stable to the hydrolysis conditions, the amount determind, as above, could not be a reliable indicator of the original loading of the FMOC tag on the polymeric reagent I. Elemental analysis was attempted as an alternative approach. The data for the C, H, N analyses of the FMOC reagent was consistent with the theoretical value calculated[15].

Preparation, isolation and characterization of external standard of cadaverine

The authentic, external standard of fluorenylmethyl cadaverine was prepared in-house and characterized. This was prepared by reacting 9-fluorenylmethyl chloroformate (18:4 mmol) with cadaverine (9.2 mmol) in 15 ml chloroform at room temperature for 30 min. The organic solvent was then evaporated under vacuum. The derivative of cadaverine was isolated by preparative HPLC conditions as follows. Column: Waters μBondapak C_{18} reversed-phase, 30 cm × 7.8 mm I.D. (semipreparative); mobile phase: acetonitrile–water (70:30); flow-rate: 2.5 ml/min; retention time: 14.4 min.

The physical and spectral properties for this compound have been reported previously, and the reader is referred to the literature for further details. Since all of the analytical and structural data were consistent with the expected structure, the characterized compound was then used as an external standard to determine percent reactions with the polymeric reagent for cadaverine, putrescine, and 1,7-diaminoheptane, under different reaction conditions. These data were reported previously[15]. Since a standard addition technique was used here to quantitate for cadaverine and putrescine in urine, starting with the base polyamines, no external standards were needed, other than to qualitatively identify the presence of such polyamines in urine after derivatization.

Procedure for derivatization of polyamines

The step-by-step procedure for derivatization of both polyamines, cadaverine and putrescine, has been reported elsewhere[15]. Approaches leading to these optimization conditions were similarly described. The derivatization reaction for a typical polyamine with this reagent is shown in Fig. 2. The HPLC–UV/fluorescence conditions used for the determination of the polyamine derivatives from urine were: acetonitrile–water (70:30), flow-rate 1.5 ml/min, 100 mm × 8 mm I.D. Radial-Pak Resolve C_{18} column, 5 μm, used in an RCM-100 module, fluorescence at 275/315 nm, UV at 254 nm.

Optimization of excitation and emission wavelengths

The excitation and emission wavelengths of the FMOC derivatives of amino acids have been reported to be 264 and 340 nm[30]. These wavelengths were optimized using flow injection analysis (FIA) by changing the emission wavelength, while holding the excitation wavelength constant. Knowing the optimum emission wavelength, the excitation wavelength was further optimized. The optimal excitation and emission wavelengths were 275 and 315 nm for the polyamine derivatives of the polymeric benzotriazole fluorenylmethyl reagent, I.

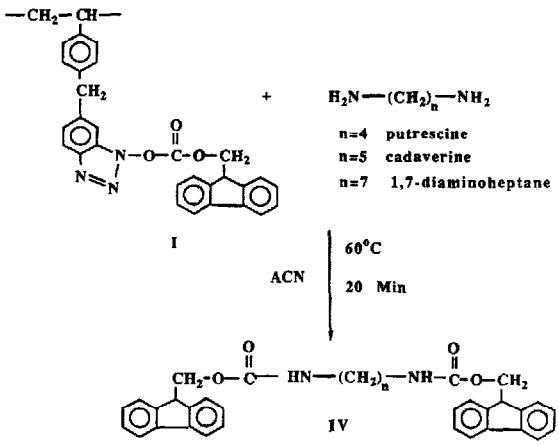

Fig. 2. The derivatization reaction of typical polyamines with the polymeric benzotriazole fluorenylmethyl (FMOC) reagent. ACN = Acetonitrile.

Urine sample preparation and derivatization of polyamines

Sample preparation and collection. It was necessary to have a bottle of about 2 l capacity. The bottle was clean, and toluene (as preservative) was added before collection was started. At a convenient time in the morning, the bladder was emptied and the specimen was discarded. This voiding represented urine produced by the kidneys before the period of collection. Thereafter all urine voided up to and including the time corresponding to that of the discarded specimen of the previous morning was placed in the bottle, which was kept in a refrigerator. Analyses were carried out as soon as possible after collection of the specimen.

Measurement of volume. Since the amount of a polyamine in the whole specimen was found by calculation from the amount determined in an aliquot, it was necessary to know the exact volume of the 24-h specimen. Hence the volume was carefully measured before any other determination was made.

Procedure for hydrolysis and derivatization of urine samples. A urine sample was collected during a 24-h period. The total volume was 1.8 l. To develop a suitable procedure for derivatization of 24-h urine, several factors had to be taken into consideration. The overall scheme for hydrolysis and derivatization of a typical urine sample was as follows: urine (or spiked urine) → hydrolysis → centrifuge → neutralization → derivatization → dilution → injection into HPLC system.

Since the polyamines were present in human urine predominantly as conjugates, it was necessary to hydrolyze the sample prior to analysis in order to liberate any polyamines from their conjugates. Most reports indicate total polyamines present, as free and conjugates in urine. A modified procedure from the literature is described as follows[31]. A 5 ml 24-h urine sample was added with 1.2 ml conc. hydrochloric acid in a reaction vial. After hydrolysis for 16 h at 110°C in an oil bath, the hydrolysate was neutralized with 6 *M* sodium hydroxide and the pH was adjusted to 9. The solution was centrifuged at 4000 rpm for 10 min and any precipitate was discarded. The clear solution was condensed to a volume of 1.5 ml. This aqueous solution (50 μl) was derivatized with the polymeric benzotriazole FMOC reagent in a

disposable Pasteur pipette at 60°C for 30 min. After reaction, the product was washed from the reaction cartridge with acetonitrile to a volume of 1 ml and 20 μl was injected into the HPLC system.

Method for determining creatinine levels in human urine. Most methods for determining creatinine are based on the Jaffe reaction described in 1886. Creatinine, in this reaction, reacts with alkaline picrate to form an amber-yellow solution that is measured photometrically. The nature of the substance formed is thought to be a salt of creatinine, picric acid, and sodium hydroxide[32].

Reagents used. The reagents used are sodium hydroxide, 0.75 *M* and picric acid. Since picric acid is explosive when it is dry, a certain amount of water was added. To determine the concentration of picric acid, a standard solution of sodium hydroxide (0.75 *M*) was used to titrate against a solution of picric acid. The concentration of picric acid was calculated to be 0.056 *M*. Creatinine was used as the working standard. Various concentrations (5, 10 and 20 ppm) of creatinine solutions were prepared by dissolving creatinine standard (free base, purchased from Sigma) in 0.1 *M* hydrochloric acid.

Procedure. Dilute 5 ml of 24-h urine to 500 ml in a volumetric flask and mix well. Pipet 6 ml of the diluted urine into a test tube and add 2 ml of 0.056 *M* picric acid and 2 ml of 0.75 *M* sodium hydroxide, mix well. Treat 6 ml of distilled water (this was used as a blank) and 6 ml of working standard creatinine in the same way. Allow to stand for 15 min and read spectrophotometrically within next 30 min.

RESULTS AND DISCUSSION

Percent derivatizations of cadaverine as a function of solvent

We had previously shown that of all the solvents tested, acetonitrile offered the highest percent derivatizations under optimal time and temperature conditions. More specific conditions for both derivatizations and HPLC for the polyamine standards have been reported elsewhere[15]. There was a significant decrease in percent derivatization as the solvent was changed from acetonitrile to hexane to water (72.1 ± 5.8%, 39.1 ± 2.5% and 7.1 ± 0.5%, respectively, average ± S.D., n = 9–11). The fact that an aqueous solution provided the lowest percent reactions of all was significant, since the polymeric reagent was to be used for polyamines in aqueous–biological fluids. The overall reproducibility of the derivatizations in water was excellent, with a relative standard deviation (R.S.D.) of about 7%, less than the R.S.D. in some other solvents. Thus, even for a low percent derivatization, reproducibility can be acceptable. However, using water as the solvent had two major advantages: (i) convenience; and (ii) no extraction was needed. As will be seen, even with this reduced reactivity, the extreme sensitivity of the final derivatives by fluorescence overcame a potential reactivity deficiency. Though most analysts would prefer percent derivatizations that approach 100% at all times, this becomes a moot point if the final derivatives formed can be detected at the trace levels needed, even with a 1% derivatization. That was exactly the case with polyamines in urine using the polymeric fluorescent derivatizing reagent, I.

Using aqueous samples, it is likely that water reacts as a nucleophile and causes hydrolysis of the polymeric reagent and thus bleeding. However, based on our studies, we have not seen excess bleeding using water as a solvent as compared to that

using acetonitrile. More strenuous conditions are required to hydrolyze this polymeric reagent.

Analysis of cadaverine and putrescine in 24-h urine samples

Usually, 24-h samples of urine are used in any quantitative analysis of polyamines in urine. Quantitative determinations of the polyamines of random voiding are of no value or significance in the interpretation of metabolic processes. it is only when all the urine produced by the kidneys over a known period of time is analyzed, that conclusions can be drawn as to what the body is doing with various foodstuffs. A 24-h sample is usually preferable. This was done here, and a literature modified procedure was perfected for collection of urine, storage of urine, hydrolysis, and pH neutralization (see Experimental).

The final urine solution after hydrolysis was derivatized in one of two ways: (1) by adding a small aliquot of a known volume of the urine to a fixed amount of the polymeric reagent in a disposable pipette (see Experimental and ref. 15); or (2) by adding a fixed amount of the polymeric reagent to an aliquot solution of the hydrolyzed and neutralized urine, *en toto*. That is, derivatizations could be successfully performed just by adding some of the polymeric reagent to the hydrolyzed urine, followed by mild heating (60 °C) for a short period of time (10–20 min). This probably represented the simplest derivatization method for any bioorganic found in urine, since it did not require any liquid–liquid extractions nor solid phase removal of the analyte prior to derivatization.

A chromatogram of an unspiked, hydrolyzed urine sample after derivatization is shown in Fig. 3. The HPLC conditions involved: acetonitrile–water (70:30) isocratic; 1.5 ml/min; Waters μBondapak C_{18} reversed-phase, 30 cm × 3.9 mm I.D.; Hitachi Model F1000 fluorescence spectrophotometer; sensitivity setting: 1.0/10 mV; excitation (ex)/emission (em) wavelengths: 275 nm/315 nm. Other HPLC mobile phase conditions involved acetonitrile–water (80:20), isocratic, also at 1.5 ml/min flow-rate. Quite different retention times were obtained just by altering the acetonitrile–water ratio. An authentic standard of the FMOC cadaverine derivative was used to identify the cadaverine peak in the urine sample chromatograms, under two HPLC conditions. The peak areas of cadaverine and putrescine derivatives under each HPLC condition appeared approximately the same. Additional confirmation for the two analytes in question came from the standard addition spiking studies, which resulted in increases in peak heights and areas for only two of the peaks present in Fig. 3. The recoveries of cadaverine and putrescine after the above hydrolysis conditions have been reported to be nearly 100%[33]. We did not perform separate polyamine recovery studies here, but have assumed our recoveries equal to those of the literature.

It did not seem likely that the peaks assigned to cadaverine and putrescine could have been due to interferences or artifacts of the analytical method. Such potential interferences would have had identical retention times to the FMOC-polyamine derivatives, under two different reversed-phase conditions, and to have identical fluorescence responses at these particular wavelengths (ex/em) to those of the FMOC-polyamines. In addition, the potential interferents would have had to have been present, underivatized or after derivatization, at levels exactly equal to those of the incurred polyamines, so that they would yield standard addition plots with excellent coefficients of linearity. It seems highly unlikely that there would have been two

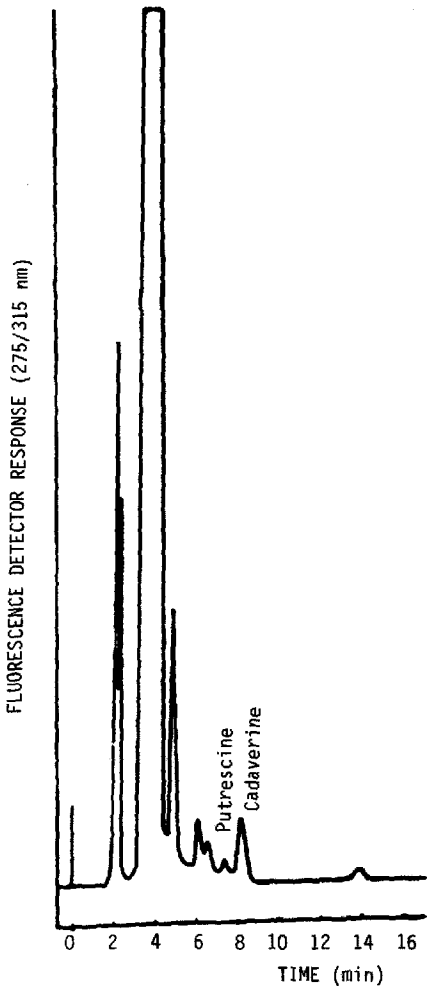

Fig. 3. Chromatogram of an unspiked, hydrolyzed urine sample after derivatization, showing the presence of cadaverine and putrescine. HPLC conditions involved: acetonitrile–water (70:30) isocratic; 1.5 ml/min; Waters μBondapak C_{18} reversed-phase, 30 cm × 3.9 mm I.D.; Hitachi Model F1000 fluorescence spectrophotometer; sensitivity setting: 1.0/10 mV; excitation/emission wavelengths: 275 nm/315 nm.

interferents that would meet all of these criteria and thereby provide false positives in both cases that agree so well with known, incurred levels of these two polyamines, as below.

Determination of the creatinine excretion

Since polyamine excretion is more nearly constant from day-to-day when expressed in mg/g creatinine, and to make it possible to make a comparison with reported values, it was essential to determine the creatinine extracted in urine. This followed a standard assay for creatinine described elsewhere[33].

Normal value of creatinine coefficient. It has been known for more than 50 years

that the 24-h excretion of creatinine per kilogram of body weight is approximately constant[33]. When the kidney function is normal, creatinine synthesis and excretion of creatinine in the urine is directly proportional to the muscle mass, and is independent of the physical effort from hour-to-hour. For a given individual it is approximately constant from day-to-day. It is possible therefore to control the reliability of the urine collection by determination of the creatinine. The following normal values[33] of creatinine coefficient can be applied for the creatinine excretion: males: 20–26 mg of creatinine per kg body weight per 24 h; women: 14–22 mg of creatinine per kg body weight per 24 h.

Calculation of creatinine in 24-h urine. UV spectra for a urine sample and creatinine standards of various concentrations (5, 10 and 20 ppm) were obtained in order to quantitate creatinine in urine. A calibration curve was obtained by plotting absorbance at 490 nm *vs.* concentration of creatinine. Based on the calibration curve of creatinine standard, the amount of creatinine excreted in the urine sample was 1.65 g/24 h, which was within the range of normal values: from the normal value of creatinine coefficient for males and the body weight of the subject (142 lbs.; 64.545 kg), the normal value of creatinine excreted could be calculated as 1.29–1.68 g/24 h.

Determination of cadaverine and putrescine levels in 24-h urine

Standard addition method. In order to determine the levels of cadaverine and putrescine present in the 24-h urine sample, we used the standard addition method, lacking an authentic standard of the FMOC putrescine derivative. We also did not know the exact percent derivatizations for each polyamine in a urine matrix. Thus, the simplest approach appeared to be that of standard addition. A urine sample was spiked with both polyamines of different levels (24 and 46 ppm for putrescine, and 8 and 46 ppm for cadaverine). These levels were chosen based on the literature values for these polyamines in normal, healthy human subjects. The same hydrolysis and derivatization procedures as described before were followed. A urine sample spiked with 24 ppm putrescine, 8 ppm cadaverine, and 1,7-diaminoheptane (internal standard), followed by derivatization, is shown in Fig. 4, with conditions indicated. Though there was no 1,7-diaminoheptane present in the normal urine sample, it was also spiked into the urine just to show the possible elution order and baseline separation for three very similar polyamines. Standard addition plots were obtained by plotting peak height *vs.* concentration spiked. The regression line equations and correlation coefficients are shown in Table I.

Cadaverine and putrescine levels in a urine sample were calculated based on the line equations for the standard addition plots.

For putrescine: $y = 2.85x + 3.164$
when $y = 0$, $x = 1.1$ ppm
1.1 ppm · 1800 ml/1.65 g creatinine = 1.20 mg/g creatinine.

For cadaverine: $y = 1.394x + 2.574$
when $y = 0$, $x = 1.8$ ppm
1.8 ppm · 1800 ml/1.65 g creatinine = 1.96 mg/g creatinine.

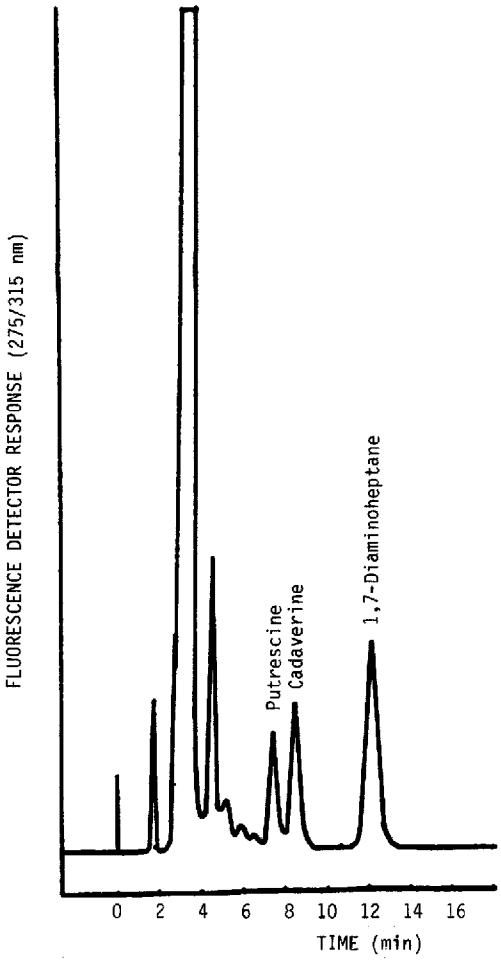

Fig. 4. Chromatogram for a typical hydrolyzed and spiked urine sample after derivatization, showing the presence of the cadaverine and putrescine peaks at higher levels. HPLC conditions as in Fig. 3.

TABLE I

STANDARD ADDITION LINE EQUATIONS AND CORRELATIONS FOR POLYAMINES IN URINE

m = Slope of the line, b = the y-intercept, r = the correlation coefficient: $y = mx + b$ for a straight line equation, as here.

Compound	*m*	*b*	*r*
Putrescine	2.850	3.164	0.999
Cadaverine	1.394	2.574	0.998

Comparison of determined polyamine values to reported ones. It has been reported that cadaverine and putrescine levels in cancer patients were significantly higher than those in normal subjects[34]. Polyamine values are generally expressed in mg/g creatinine, because polyamine excretion is more nearly constant from day-to-day when expressed in mg per day. Different expressions have been used such as μg/ml, mg/24 h, and mmol/mg creatinine. The cadaverine and putrescine levels in normal urines are 0.26–2.06 and 1.06–2.92 mg/g creatinine, respectively. However, for cancer patients the levels of putrescine and cadaverine are 50–85% and 21–31% exceeding the normal upper limits. Our determined values of cadaverine and putrescine (1.96 ± 0.07 and 1.20 ± 0.05 mg/g creatinine ± S.D., $n = 9$), using a standard addition method, were in the ranges of reported values for healthy, normal subjects. The detection limits for the determination of the polyamines, cadaverine and putrescine, in urine were 21 pmol and 24 pmol, respectively.

Suggestions on possible automation of the overall polymeric derivatization. Automated pre-column derivatizations have the same basic requirements and limitations as non-automated techniques. However, automation generally increases throughput and decreases operator attendance and analysis cost. In principle, automation should have been widely employed and described for derivatization in LC, but quite the opposite has been the case. Perhaps the necessity of extra instrumentation, method codification and sophistication, or method development has dissuaded many from employing this approach. As the hardware and software become less expensive and easier to use, automated techniques should become prevalent.

Various commerical firms have developed solid phase extraction tubes which are filled with silica-based, monomerically bonded packings, and have been used for sample cleanup[35]. The polymeric benzotriazole reagent could be easily packed into such tubes in the same manner as the bonded phases used today for cleanup. The Visiprep™ solid phase extraction vacuum manifold developed, *e.g.*, by Supelco, contains a built-in vacuum bleed valve and gauge, a heavy duty glass basin, chemical resistant cover and seals, stainless-steel solvent guide needles, adjustable screw-type valves built into the cover, a collection rack, and a plate for autosampler vials. This device allows one to accurately adjust the vacuum, and thus control the time of a solid phase derivatization reaction and final filtration of derivatized sample. The polypropylene collection vessel rack accommodates autosampler and vials, and thus injection could be automated[35]. Similar solid phase extraction devices (*e.g.* Baker-10 SPE System by J. T. Baker) could be used in the same manner for the purpose of automation of derivatizations.

CONCLUSIONS

Polystyrene-bound benzotriazole reagents are feasible and practical for the off-line derivatization of nucleophiles, such as simple amines and polyamines, in connection with HPLC for trace analysis. In addition to the advantages usually possible for related supported reagents in heterogeneous reactions, these particular polymeric reagents have exhibited higher derivatization yields and lower detection limits. This has been a direct result of the tag/label selected. The choice of a label that can impart multiple detection capabilities, such as UV–fluorescence for the fluorenyl reagent or UV–electrochemical detection for the *o*-acetylsalicyl reagent, has further expanded

the overall utility and applicability of such newer reagents for improved detection in HPLC.

Detection limits for the derivatives were excellent, usually 2–3 orders of magnitude lower than for the starting polyamines. Derivatization yields were high, and reactions required mild conditions (low temperature, short time, mild solvents). Derivatizations with the polymeric fluorenyl reagent for polyamines have occurred at room temperature within 10 min, in mild solvents (acetonitrile, dioxane, etc.). The derivatives were formed in yields of about 50% using the above conditions. Reactions were also successful, though with lower percent conversions, in aqueous or aqueous–organic solutions. Derivatizations have been possible simply by adding the polymeric reagent to the aqueous sample solution, derivatizing, filtering off the unused reagent, and injecting the derivatized solution into the HPLC. Sample work-up and extraction of the bioorganics of interest, such as polyamines, have been greatly reduced over all current approaches. Polyamine levels in urine samples of normal subjects or cancer patients can now be quantitated using this approach, with automation entirely feasible. Further applications to serum, plasma, and tissue samples should prove just as successful.

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REFERENCES

1 I. S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, (*Chromatographic Science Series*, Vol. 34), Marcel Dekker, New York, 1986.
2 I. S. Krull, S. T. Colgan and C. M. Selavka, in P. R. Brown and R. A. Hartwick (Editors), *High Performance Liquid Chromatography*, Wiley-Interscience, New York, 1988, in press.
3 S. T. Colgan and I. S. Krull, in I. S. Krull (Editor), *Reaction Detection in Liquid Chromatography*, Marcel Dekker, New York, 1986, p. 227.
4 J. M. Rosenfeld and R. A. McLeod, in D. J. Harvey (Editor), *Marihuana '84, Proceedings of the Oxford Symposium on Cannabis*, IRL Press, Oxford, England, 1984, p. 151.
5 L. Nondek, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 282 (1983) 141.
6 H. Jansen, U. A. Th. Brinkman and R. W. Frei, *Chromatographia*, 20 (1985) 453.
7 I. S. Krull and E. P. Lankmayer, *Am. Lab. (Fairfield, Conn.)*, May (1982) 18.

8 I. S. Krull, K.-H. Xie, S. Colgan, T. Izod, U. Neue, R. King and B. Bidlingmeyer, *J. Liq. Chromatogr.*, 6 (1984) 605.
9 K.-H. Xie, S. Colgan and I. S. Krull, *J. Liq. Chromatogr.*, 6 (S-2) (1983) 125.
10 I. S. Krull, S. Colgan, K.-H. Xie, U. Neue, R. King and B. Bidlingmeyer, *J. Liq. Chromatogr.*, 6 (1983) 1015.
11 S. T. Colgan, I. S. Krull, U. Neue, A. Newhart, C. Dorschel, C. Stacey and B. Bidlingmeyer, *J. Chromatogr.*, 333 (1985) 349.
12 S. T. Colgan, I. S. Krull, C. Dorschel and B. Bidlingmeyer, *Anal. Chem.*, 58 (1986) 2366.
13 T.-Y. Chou, S. T. Colgan, D. M. Kao, I. S. Krull, C. Dorschel and B. Bidlingmeyer, *J. Chromatogr.*, 367 (1986) 335.
14 S. T. Colgan, I. S. Krull, C. Dorschel and B. Bidlingmeyer, *J. Chromatogr. Sci.*, (1988) in press.
15 C.-X. Gao, T.-Y. Chou, S. T. Colgan, I. S. Krull, C. Dorschel and B. Bidlingmeyer, *J. Chromatogr. Sci.*, 26 (1988) 449.
16 M. M. Abdel-Monem and J. L. Merdink, *J. Chromatogr.*, 222 (1981) 363.
17 C. E. Prussak and D. H. Russell, *J. Chromatogr.*, 229 (1982) 47.
18 T. Takagi, T. G. Chung and A. Saito, *J. Chromatogr.*, 272 (1983) 279.
19 M. Mach, H. Kersten and W. Kersten, *J. Chromatogr.*, 223 (1981) 51.
20 T. Skaaden and T. Greibrokk, *J. Chromatogr.*, 247 (1982) 111.
21 H. Veening, W. W. Pitt, Jr. and G. Jones, Jr., *J. Chromatogr.*, 90 (1974) 129.
22 M. Kai, T. Ogata, K. Haraguchi and Y. Ohkura, *J. Chromatogr.*, 163 (1979) 151.
23 N. E. Newton, K. Ohno and M. M. Abdel-Monem, *J. Chromatogr.*, 124 (1976) 277.
24 F. L. Vandemark, G. J. Schmidt and W. Slavin, *J. Chromatogr. Sci.*, 16 (1978) 465.
25 N. D. Brown, R. B. Sweet, J. A. Kintzios, H. D. Cox and B. P. Doctor, *J. Chromatogr.*, 164 (1979) 35.
26 B. Brossat, J. Straczek, F. Belleville, P. Nabet and R. Metz, *J. Chromatogr.*, 277 (1983) 87.
27 C.-X. Gao, *M.S. Thesis*, Northeastern University, Boston, MA, 1986.
28 R. Kalir, A. Warshawsky, M. Fridkin and A. Patchornik, *J. Biochem.*, 59 (1975) 55.
29 L. A. Carpino and G. Y. Han, *J. Org. Chem.*, 37 (1972) 3404.
30 R. Cunico, A. G. Mayer, C. T. Wehr and T. L. Sheehan, *BioChromatography*, 1 (1986) 6.
31 F. L. Vandemark, G. J. Schmidt and W. Slavin, *J. Chromatogr. Sci.*, 16 (1978) 465.
32 I. Greenwald, *J. Biol. Chem.*, 80 (1928) 103.
33 R. Richterich, *Clinical Chemistry: Theory and Practice*, Academic Press, New York, London, 1969, p. 73.
34 K. Fujita, T. Nagatsu, K. Shinpo, K. Maruta, R. Teradaira and M. Nakamura, *Clin. Chem.*, 26/11 (1980) 1577.
35 *Supelco Chromatography Supplies*, Supelco, Bellefonte, PA, Catalog No. 26, 1988, p. 216.