Gas Chromatography Mass Spectrometry Analysis of Polyamines Using Deuterated Analogs as Internal Standards

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Quantitative analyses of subnanomole quantities of the polyamines putrescine, cadaverine, spermidine and spermine by the gas chromatography mass spectrometry technique of selected ion monitoring are complicated by effects of the chromatographic column. These effects include sample retention by the column and chromatographic band broadening, and are sufficiently serious that spermine (as the trifluoroacetylated derivative) was not detectable at levels below 50 picomoles. Because the chromatographic behaviors of the four polyamines vary, quantiative analysis using a single internal standard is not feasible. The deuterated polyamine analogs, putrescine-$^2H_4$, cadaverine-$^2H_4$, spermidine-$^2H_6$ and spermine-$^2H_8$, have been synthesized and used to accomplish quantiative analyses of the corresponding isotopically natural abundance polyamines to the one picomole level. This enhancement in analytical sensitivity is accomplished by use of large excesses (≥100 picomoles) of the deuterated analogs to improve chromatographic band profiles. The use of such large molar excesses of deuterated analogs for selected ion monitoring analyses is possible because their electron impact spectra exhibit high mass ions {$[M-F_3C]^+$, $[M-F_3CCO]^+$} which possess ≥4 deuterium atoms and have intensity ratios ($I_{^2H(max)}$: I_H) of 500 or greater.

INTRODUCTION

Spermine was first isolated (from human semen) by Lewenhoeck in 1677.[1] During the three centuries since this discovery, the naturally occurring polyamines, spermine and spermidine, and the related diamines, putrescine and cadaverine, have been subject to periodic and diverse investigations.[2–5] Numerous recent studies indicate functional roles for polyamines† in processes of cell growth and cell proliferation,[2,4,5] perhaps by interaction with nucleic acids.[6–8] Interest in highly sensitive and specific methods for quantitative determination of levels of individual polyamines has been greatly stimulated by the demonstration[9] that urinary excretion of polyamines is abnormal in cancer sufferers.[9–15]

A number of new procedures for measurement of polyamine levels based on radioimmunoassay,[15] ion exchange,[14,16,18] thin-layer,[19,20] gas[22–25] and high pressure liquid[26] chromatographies have been reported. Mass spectrometry has been used as a method of detection either in combination with gas chromatography[22,23,25] or without prior chromatographic separation.[6] In this report we describe the development of a gas chromatography mass spectrometry (g.c.m.s.) method, based on the technique of selected ion monitoring (s.i.m.) and utilizing deuterated analogs, which permits quantitative analyses of spermine, spermidine, putrescine and cadaverine to the one picomole level.

Putrescine	$H_2NCH_2CH_2CH_2CH_2NH_2$
Cadaverine	$H_2NCH_2CH_2CH_2CH_2CH_2NH_2$
Spermidine	$H_2NCH_2CH_2CH_2CH_2NHCH_2CH_2 \cdot CH_2NH_2$
Spermine	$H_2NCH_2CH_2CH_2NHCH_2CH_2CH_2 \cdot CH_2NHCH_2CH_2CH_2NH_2$

†The term 'polyamines' is used to include the naturally occurring diamines 1,3-diaminopropane, putrescine and cadaverine as well as spermine and spermidine.

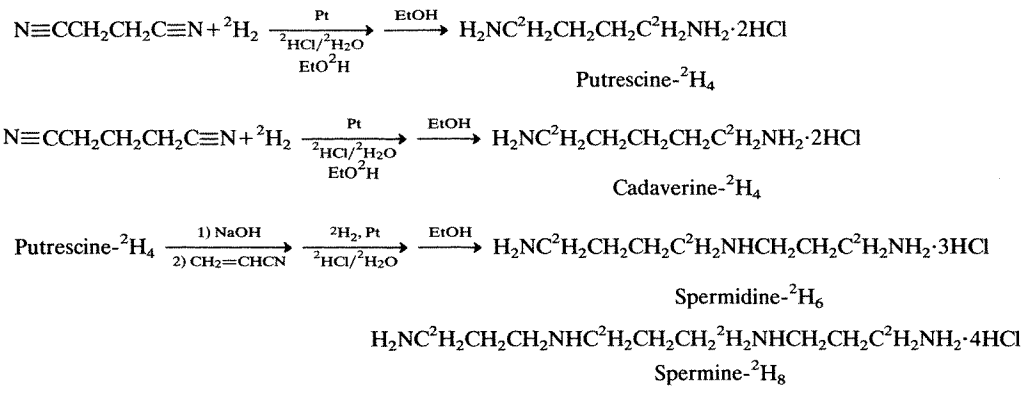
EXPERIMENTAL

Synthesis of deuterium labeled internal standards

The diamines putrescine-$^2H_4$‡ and cadaverine-$^2H_4$ were prepared by catalytic deuteration from succinonitrile and glutaronitrile, respectively (Scheme 1). A typical reaction involved dissolving 0.94 g (10 mmol) of glutaronitrile in 20 ml of *O*-deuteroethanol (95% solution in deuterium oxide, 99% ^2H) and adding 3 ml of deuterium chloride (37% solution in deuterium oxide, 99% ^2H) and 100 mg of platinum oxide. This mixture was shaken under 25–30 p.s.i. of deuterium gas (99.5% ^2H) until gas uptake ceased. The solution was filtered free of platinum, the solvents were removed under vacuum and the white solid residue was crystallized from 100% ethanol to yield 0.96 g (54%) of cadaverine-$^2H_4$ dihydrochloride, m.p. 258–260 °C (lit. m.p. 255 °C,[29] 275 °C[30]).

Deuterated analogs of spermidine and spermine were prepared by the addition of acrylonitrile to putrescine-$^2H_4$ followed by the addition of deuterium to the nitrile using a platinum catalyst. To a suspension of 0.16 g (1.0 mmol) of putrescine-$^2H_4 \cdot 2HCl$ in 20 ml of ethanol

‡Putrescine[27] and spermine[28] labeled with tritium have been prepared by a similar procedure.



Scheme 1. Synthetic schemes for the preparation of putrescine-$^2H_4$, cadaverine-$^2H_4$, spermidine-$^2H_6$ and spermine-$^2H_8$.

was added 0.2 ml of 3.8 N aqueous NaOH. After solution was effected, 60 μl of acrylonitrile was added slowly. After the mixture was stirred at room temperature for 24 h, 2.0 ml of 6 N HCl was added and the solvents were removed completely under reduced pressure. The solid residue was dissolved in 15 ml of deuterium oxide, 1.0 ml of 37% deuterium chloride (in deuterium oxide) and 50 mg of platinum oxide were added, and the mixture was shaken under 30 p.s.i. of deuterium for 36 h. The catalyst was removed by filtration, the solvents were evaporated under reduced pressure and the solid residue was dissolved and diluted to 50 ml using 1.0 N HCl. A 50 μl aliquot was combined with a 50 μl aliquot from a solution of the four unlabeled polyamines, each 1.0 mM in concentration. The combined aliquots were dried and derivatized using trifluoroacetic anhydride (TFAA). The sample was examined by s.i.m. analysis using the $[M-CF_3]^+$ ions (*m/e* 211 and *m/e* 215 for putrescine-$^2H_0$ and -$^2H_4$, *m/e* 364 and *m/e* 370 for spermidine-$^2H_0$ and -$^2H_6$, and *m/e* 517 and *m/e* 525 for spermine-$^2H_0$ and -$^2H_8$). As determined by the $^2H/^1H$ peak height ratios, the concentrations of the deuterated compounds in aqueous solution were 5.2 mM, 7.3 mM and 2.5 mM for putrescine-$^2H_4$, spermidine-$^2H_6$ and spermine-$^2H_8$, respectively. This solution was used for subsequent experiments without further separation. Impurities present, as determined by gas chromatography, did not exceed 5% and none of them exhibited any of the high mass ions $[M-CF_3]^+$ used for s.i.m. analysis.

Derivatization

Trifluoroacetylation. Aliquots from 1.0 N HCl solutions of the polyamines (prepared by dissolution of weighed samples of the hydrochloride salts, except for the previously mentioned solution of putrescine-$^2H_4$, spermidine-$^2H_6$ and spermine-$^2H_8$) were transferred to a 0.3 ml conical vial, the solvent was evaporated under a stream of nitrogen and 100 μl of TFAA was added. After sealing the vial with a Teflon lined screw cap, the sample was sonicated for 10–15 min. The excess TFAA was removed under a stream of nitrogen and a known volume of methylene chloride was added prior to g.c.m.s. analysis. This procedure adds one trifluoroacetyl group to each of the primary and secondary amines. The spectra of the unlabeled derivatives have been published[22] and the spectra of the spermidine-$^2H_0$ and -$^2H_6$ derivatives are shown in Fig. 1.

Trimethylsilylation. Aliquots from 1.0 N HCl solutions of the polyamines were transferred to a 0.3 ml conical vial and the solvent was evaporated under a stream of nitrogen. Silylation was achieved using a 2:2:1 mixture of pyridine, *N*,O-bis-(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane and sonication for 10–15 min. This procedure resulted in replacement of all amino hydrogens with trimethylsilyl groups.

Analyses of deuterium incorporation into polyamine analogs. The fragment ion $[F_3CCONH{=}CH_2]^+$ (*m/e* 126), observed in the spectrum of each of the trifluoroacetylated polyamines, is convenient for assessing the effectiveness of the platinum catalyzed deuteration reactions. Electron bombardment of either trifluoroacetylated putrescine-$^2H_4$ or cadaverine-$^2H_4$ produced ions corresponding to this fragmentation at *m/e* 126, *m/e* 127 $[F_3CCONH{=}CH^2H]^+$ and *m/e* 128 $[F_3CCONH{=}C^2H_2]^+$ which were measured by s.i.m. techniques. The percentages of deuterium incorporation (Table 1) were calculated from the relative intensities of these ions using the equation

$$\%\ ^2H\ \text{Incorporation} = \frac{(2\,I_{m/e\,128} + I_{m/e\,127}) \times 100\%}{2(I_{m/e\,126} + I_{m/e\,127} + I_{m/e\,128})}$$

Other ion intensity ratios ($I_{^2H(max)}/I_H$) for each of the deuterated polyamine analogs were obtained by s.i.m. of the $[M-F_3C]^+$ fragment ions.

Table 1. Relative ion intensities of deuterium and protium forms of ions at low and high mass (as determined by s.i.m. analysis)

	m/e 126	*m/e* 127	*m/e* 128	% ^2H	$^2H_{max}/H$
Putrescine-$^2H_4$	1	6	62.5	94.2	>580[a]
Cadaverine-$^2H_4$	1	3.3	27	91.2	>900[b]
Spermidine-$^2H_6$	1		19.6		>2000[c]
Spermine-$^2H_8$	1		14.8		>760[d]

[a] $\frac{m/e\ 215}{m/e\ 211}$ [b] $\frac{m/e\ 229}{m/e\ 225}$ [c] $\frac{m/e\ 370}{m/e\ 364}$ [d] $\frac{m/e\ 525}{m/e\ 517}$

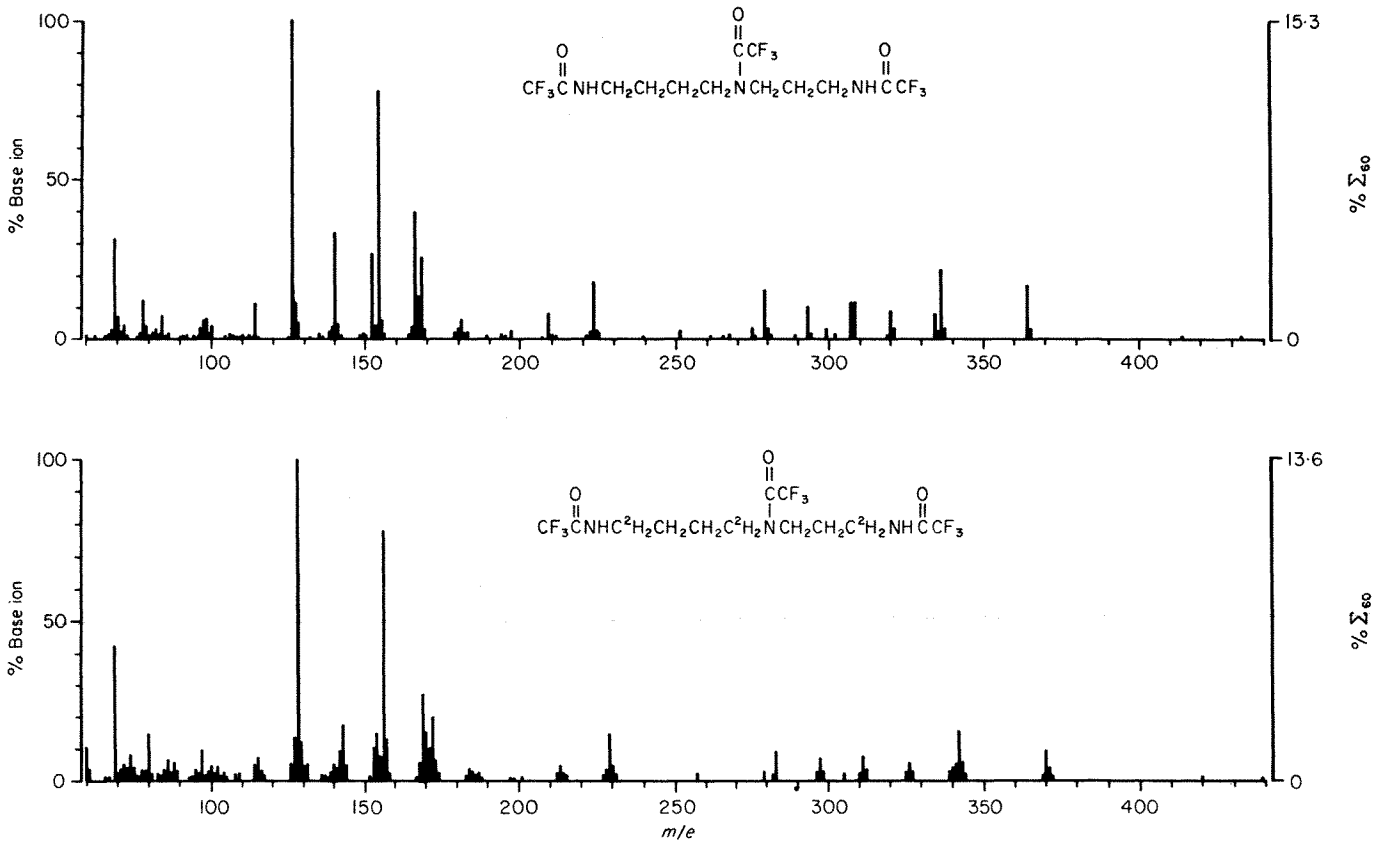


Figure 1. Mass spectra of the trifluoroacetylated derivatives of spermidine and the deuterated ($^2H_6$) analog.

Instrumentation

Mass spectral experiments were carried out using a DuPont 21-491B mass spectrometer interfaced with a Varian 2700 gas chromatograph. This system is equipped with a four channel DuPont MSID accessory for s.i.m. analyses. Outputs from the gas chromatograph flame ionization detector and the specific ion monitors were recorded on a Gould Brush 260 six channel recorder.

Most experiments utilized a 6 ft × 2 mm glass column packed with 1.5% OV-101 on 100/120 Gas Chrom Q. Other packing materials examined in an attempt to optimize peak shape and decrease column adsorption were 3% OV-17 on 80/100 Chromosorb W-HP and 3% Dexsil 300 on 80/100 Chromosorb W, AW-DMCS. These columns provided no improvement. It was later seen that a shorter column (4 ft) slightly decreased the temperatures required for elution of the polyamine derivatives without peak broadening or loss of resolution. Subsequent experiments were carried out with this column. Isothermal experiments such as those used in constructing the response curves were run at column temperatures providing short (2–3 min) retention times for the compound of interest. Using the shorter column, these temperatures were 115, 120, 180 and 260 °C for the trifluoroacetyl derivatives of putrescine, cadaverine, spermidine and spermine, respectively. The temperature program used for both the trifluoroacetyl and trimethylsilyl derivatives was 120–310 °C programmed at 15 °C min^{-1}. An s.i.m. analysis of all four polyamines using the high mass $[M-CF_3]^+$ ions was accomplished by manually switching the channels to different masses during the time available between elution of the different polyamine derivatives.

RESULTS AND DISCUSSION

The decision to employ deuterated polyamine analogs was made following an initial study in which we attempted direct g.c.m.s. analysis of polyamine trifluoroacetyl derivatives[22–24] in the subnanomole range using the homolog, diaminoheptane, as an internal standard.[23] As shown in Fig. 2(a), each of the four polyamine derivatives exhibited excellent chromatographic properties when placed on the column in quantities of 500 picomoles or greater. However, when smaller quantities of the polyamine samples are chromatographed [Fig. 2(b and c)] severe deterioration in the quality of the chromatographic behavior was observed. At the ten picomole level [Fig. 2(c)] spermine was no longer detected and the chromatographic bands for the putrescine and cadaverine derivatives had broadened and partially coalesced. Slight improvements in band shape and analytical sensitivity were attained by decreasing the residence time of the polyamine derivatives on the column. However, the chromatographic band profiles for putrescine, cadaverine and spermidine differed sufficiently to preclude accurate measurement using a single internal standard. More importantly, the analytical sensitivity for spermine remained in the high picomole range—unsuitable for our purposes. To overcome these

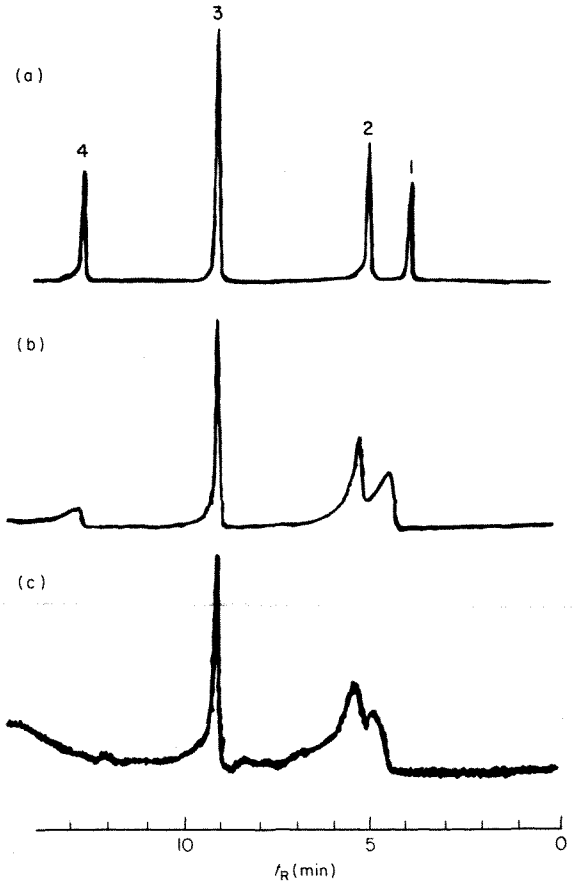


Figure 2. Effect of decreasing sample size on the chromatographic bands of the trifluoroacetylated polyamine derivatives. Selected ion recordings of the ion at *m/e* 126 $[F_3CCONH{=}CH_2]^+$ in samples containing (a) 500 picomoles, (b) 50 picomoles and (c) 10 picomoles each of the trifluoroacetylated derivatives of putrescine (**1**), cadaverine (**2**), spermidine (**3**) and spermine (**4**). The experiments utilized a 6 ft × 2 mm glass column of 3% Dexsil 300 on 80/100 Chromosorb W, AW-DMCS. The column temperature was programmed 120–330 °C at 15 °C min^{-1}.

difficulties the deuterated polyamine analogs (Scheme 1) were prepared for use both to improve the chromatographic properties of picomole quantities of biological polyamines and thereby increase analytical sensitivity, and as individual quantitative standards for each of the polyamines. Moreover, addition of deuterated analogs into biological samples prior to preanalysis processing obviates estimations of polyamine losses during sample manipulation.

Polyamine derivatization

In common with previous workers,[22–24] we have used trifluoroacetyl derivatives of the polyamines for g.c.m.s. analyses. These derivatives, which possess one trifluoroacetyl group per polyamine nitrogen, are easily prepared, relatively stable and exhibit electron bombardment fragmentation (Fig. 1 and discussion below) suitable for picomole sensitivity in s.i.m. analyses. More recently, we have prepared trimethylsilyl (TMS) derivatives. These derivatives also appear to be suitable for g.c.m.s. analysis. The spectra (Fig. 3 shows representative spectra, i.e. those of trimethylsilylated putrescine and its $^2H_4$ analog) exhibit high mass ions $[M-15]^+$ and intense fragment ions at *m/e* 174 $[CH_2{=}N(TMS)_2]^+$ suitable for s.i.m. analysis. In preliminary experiments, the putrescine and cadaverine derivatives eluted as sharp bands down to the 100 femtomole level.† However, the spermidine and spermine trimethylsilyl derivatives appear to be adsorbed by the chromatographic column to a greater degree than occurs with the corresponding trifluoroacetyl derivatives (Fig. 2).

Enhancement of analytical sensitivity using deuterated analogs

In early studies, we observed rapid deterioration of chromatographic band shapes as the amounts of polyamine trifluoroacetyl derivatives placed on the column decreased below 100 picomoles (Fig. 2). This suggested that use of deuterated analogs of the individual polyamines to maintain the total level of a polyamine derivative (natural abundance polyamine plus deuterated analog) at ≥100 picomoles would significantly enhance the sensitivity of g.c.m.s. polyamine analysis. The data shown in Fig. 4 confirm this expectation. One picomole of trifluoroacetylated putrescine was not detectable [Fig. 4(a)]. However, a one-picomole sample, to which 100 picomoles of the $^2H_4$ analog had been added, gave a well defined chromatographic band [Fig. 4(b)]. That only a small percent of this band can be attributed to the deuterated 'carrier'[32,33] was shown by the control experiment [Fig. 4(c)]. The response curve (Fig. 5, see discussion below) in which 100 picomoles of a deuterated polyamine was used to attain a linear s.i.m. response to low levels of natural abundance polyamine is another demonstration of this sensitivity enhancement.

Selection of ions for s.i.m. analyses of individual polyamines

The preliminary study (Fig. 2) established that a sample of polyamine substantially in excess of 50 picomoles was necessary to obtain chromatographic behavior suitable for quantitative analysis. Therefore, analyses at the one picomole level requires addition of 100 picomole or more of deuterated polyamine standard to serve as a chromatographic 'carrier'.[32,33] The necessity for use of such large excesses of the deuterated polyamines limits the choice of ions suitable for quantitative s.i.m. analyses. To accurately measure the intensity of an ion derived from a polyamine at isotopic natural abundance, it is necessary that the contribution to this intensity by ions derived from the deuterated internal standard be small (in a relative sense). Therefore, s.i.m. analyses using the intense fragment ion—*m/e* 128 ($^2H_2$) and 126 ($^2H_0$)—are precluded since this ion pair for all of the polyamine analogs exhibit intensity ratios of <100 (Table 1). In contrast, high mass ions $[M-F_3C]^+$ and $[M-F_3CCO]^+$ which possess all of the deuterium originally incorporated into the polyamine standards (i.e. 4–8 ^2H atoms, Scheme 1) exhibit ion intensity ratios $(I_{^2H(max)}:I_H)$ of >500 (Table 1). While these ions are

† This represents a measurable quantity [e.g. Fig. 4(b)]. The lower limit of detection previously has been reported[31] to be 15 femtomoles.

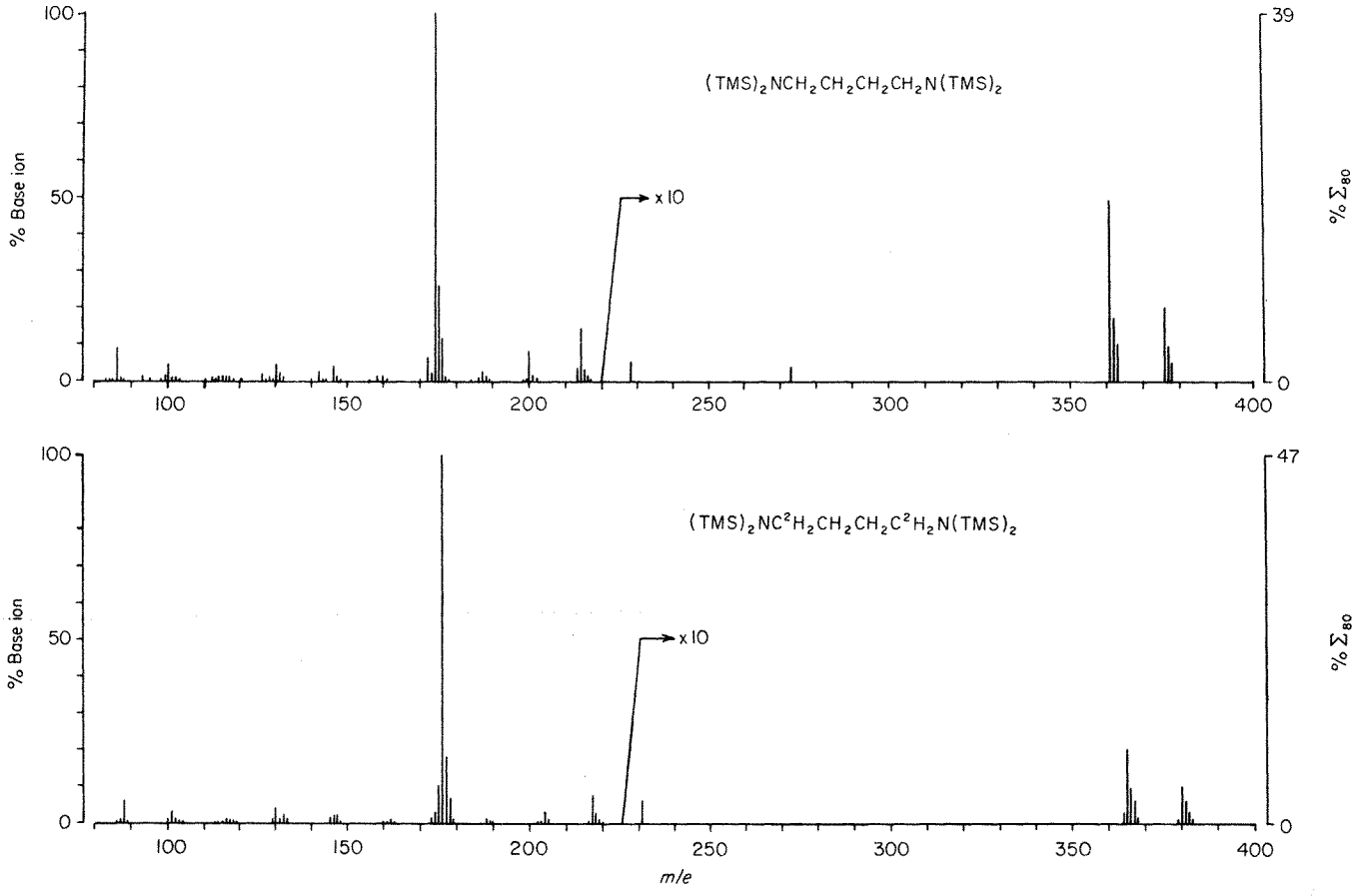


Figure 3. Mass spectra of the trimethylsilyl derivatives of putrescine and the deuterated ($^2H_4$) analog.

somewhat less intense than the lower mass base ions (*m/e* 126)† two other advantages–increased specificity for detection of the compound of interest and decreased interference from background ions–contribute to their selection for use in s.i.m. analyses of polyamines.

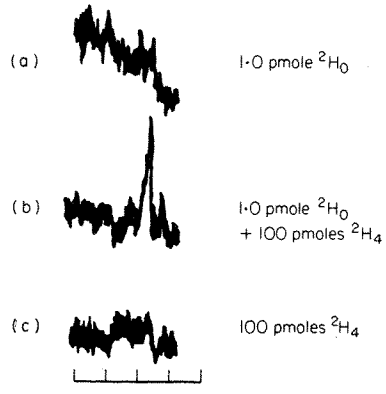


Figure 4. Sensitivity enhancement from the addition of deuterated analog. Selected ion recordings of trifluoroacetylated putrescine recording the ion at *m/e* 211 (loss of CF_3 from putrescine-$^2H_0$).

(a) 1.0 picomole of putrescine-$^2H_0$. (b) 1.0 picomole of putrescine-$^2H_0$ with 100 picomoles putrescine-$^2H_4$ added. (c) 100 picomoles of putrescine-$^2H_4$. These experiments utilized a 4 ft × 2 mm glass column of 1.5% OV-101 on 100/120 Gas Chrom Q. The column oven temperature was isothermal at 115 °C.

† Trifluoroacetylated spermine is an exception. It exhibits an ion at *m/e* 517 $[M—F_3C]^+$ which is slightly more intense than the ion at *m/e* 126.

Standard s.i.m. response curves

A criterion for a good internal standard is that it allow accurate quantitative analysis over a wide concentration range and over a wide range of molar ratios of sample to

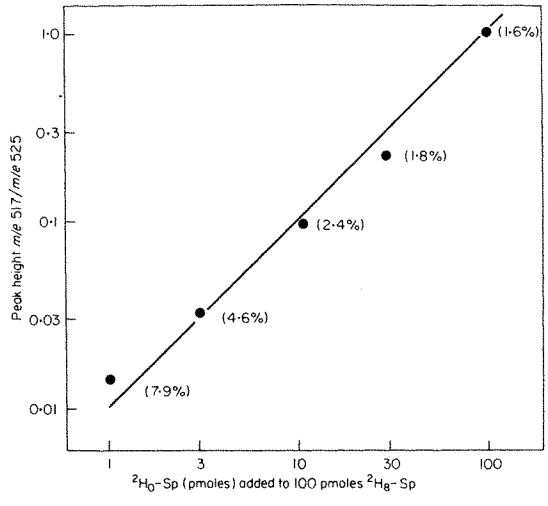


Figure 5. Response curve for trifluoroacetylated spermine-$^2H_0$: response of varied sample sizes of trifluoroacetylated spermine-$^2H_0$ in a mixture with 100 picomoles of trifluoroacetylated spermine-$^2H_8$ as determined by the peak height ratios of the $^2H_0$ to $^2H_8$ ions (at *m/e* 517 and *m/e* 525, respectively) obtained in s.i.m. analysis. Each point represents the average of four to six injections and the coefficients of variation are given in parentheses. The response is plotted on log–log coordinates and the line represents the theoretical linear response.

internal standard. Using the deuterated polyamine analogs, a linear response to polyamine levels over a range of 100 picomoles–1.0 picomole (illustrated for trifluoroacetylated spermine, Fig. 5) was attained. More importantly, this standard curve (Fig. 5) shows that linearity of response was attained over a 100-fold range in molar ratios of sample to standard. The precision of these measurements was good, with the coefficients of variation consistently below 10.

In conclusion, methodology has been described which permits analyses of four individual polyamines to the one picomole level. This degree of sensitivity, while not needed for the analysis of urinary polyamines, is essential for examination of other physiological fluids such as blood serum and cerebrospinal fluid. It is noteworthy that relatively little is known concerning spermine levels due to the apparent low concentrations of this polyamine and the absence of adequate analytical sensitivity.‡ The technique of s.i.m. analysis using deuterated analogs as internal standard should provide the sensitivity required.

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‡ The radioimmunoassay method for spermine determination[15] also is sensitive at the picomole level and has been used to generate a substantial body of data concerning spermine levels in blood sera of normal and diseased individuals.[34]