Research Article

Journal of MASS SPECTROMETRY

Received: 7 November 2008 Accepted: 20 April 2009 Published online in Wiley Interscience: 9 June 2009

(www.interscience.com) DOI 10.1002/jms.1597

A quantitative GC-MS method for three major polyamines in postmortem brain cortex

Gary G. Chen,[a] Gustavo Turecki[a]* and Orval A. Mamer[b]*

A quantitative method for putrescine (PUT), spermidine (SPD) and spermine (SPM) in homogenized postmortem human brain tissue is described that employs a novel, simple and rapid extractive derivatization with ethylchloroformate and trifluoroacetylation. These amines are metabolites of ornithine and are metabolically interconvertible in mammals. The method was developed to support an ongoing epidemiological study correlating these amines with the frequency of suicide. The isolation methodology is robust and requires less work and time than many previous methods. Analysis is by conventional electron ionization GC-MS with selected ion monitoring using a stable isotope-labeled analog for PUT and a chemical analog for SPD and SPM as internal standards. The time required for chromatographic analysis, about 20 min, is determined by the wide range of the relative volatilities of the derivatized polyamines. The method allows the quantitation of PUT down to 10 ng/g and SPD and SPM down to 100 and 1000 ng/g, respectively of wet tissue.

Keywords: polyamines; suicide; GC-MS; selected ion monitoring; derivatization; postmortem brain tissue

Introduction

Putrescine (1,4-diaminobutane; PUT), spermidine (*N*-[3-aminopropyl]-1,4-diaminobutane; SPD) and spermine (*N*,*N*′-bis[3-aminopropyl]-1,4-diaminobutane; SPM) (Fig. 1) belong to a family of low molecular weight alkyl polyamines that are ubiquitous and have critical roles in many mammalian processes, including cell proliferation and differentiation, angiogenesis, acute mental depression, aging and inflammation.[1–3] They are metabolically derived from ornithine and are interconvertible:

$$\text{Orn} \longrightarrow \text{PUT} \longleftrightarrow \text{SPD} \longleftrightarrow \text{SPM}$$

They exist as protonated cations at physiological pH. They most likely function via interactions with anionic biomolecules such as DNA, phospholipids and cellular proteins. Their biosynthesis and intracellular levels are tightly controlled by a complicated system of anabolic and catabolic pathway enzymes and these ensure that rapid polyamine-level changes can be achieved in response to specific intrinsic needs.

Many polyamine analytical methods have been developed. Most methods use HPLC[4] or GC.[5] Since these methods often rely on peak retention times for detection, specificity may be an issue because of interferences from co-eluting or overlapping peaks. Polyamine analysis of postmortem brain tissues by HPLC has been reported.[6] Since then, GC-MS has been introduced for the analysis of polyamines in several biological systems.[7,8] An efficient GC-MS method based on a two-phase extractive derivatization procedure has been used for polyamine analysis of human hair.[9] A method employing a single derivatization with *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide for the analysis of a variety of neurochemicals in rat hippocampal slices has been reported by Wood *et al*.[10] Although it is a simple and effective method, the only polyamine measured was PUT in normal rats and those treated with an organotin toxin. Electron ionization (EI) methods using GC-MS correlating all three interrelated polyamines from postmortem brain samples from suicide victims with severe depression have not been explored. In preliminary studies, we noted that positive ion electrospray of the underivatized amines extracted from tissue samples was not as sensitive by about an order of magnitude as the method described here.

Here, we present an analytical method for polyamine profiles from postmortem brain tissues based on the ethoxycarbonylation of amino groups in aqueous phase under mild alkaline conditions followed by trifluoroacetylation of the resulting urethanes in organic solvent phase at loci that possess a residual N–H bond. Selected ion monitoring (SIM) techniques were used with [2,2,3,3-$^2H_4$]-1,4-diaminobutane (PUT-D_4) as the internal standard for endogenous PUT and 1,7-diaminoheptane (DAH) as the internal standard for SPD and SPM, as deuterium labeled analogs of these were unavailable commercially. The goal of this study is to develop a method to determine the levels of these three major polyamines in postmortem cortex tissue of suicide victims. To date, we have analyzed a few hundred postmortem samples with this method.

Experimental

Chemicals and tissue samples

PUT, SPD, SPM and DAH as the free bases, derivatization grade ethyl chloroformate (ECF) and trifluoroacetic acid anhydride (TFAA) were obtained from Sigma (St. Louis, MO, USA). PUT-D_4 was a

* *Correspondence to: Gustavo Turecki, Department of Psychiatry, Douglas Hospital, 6875 LaSalle Blvd, Montreal QC, H4H 1R3, Canada. E-mail: gustavo.turecki@mcgill.ca*

Orval A. Mamer, Mass Spectrometry Facility, McGill University, 740 Dr. Penfield, Suite 5300, Montreal QC, H3A 1A4, Canada E-mail: orval.mamer@mcgill.ca

a *McGill Group for Suicide Studies, Douglas Hospital, McGill University, Montreal, Quebec, Canada*

b *Mass Spectrometry Unit, McGill University, Montreal, Quebec, Canada*

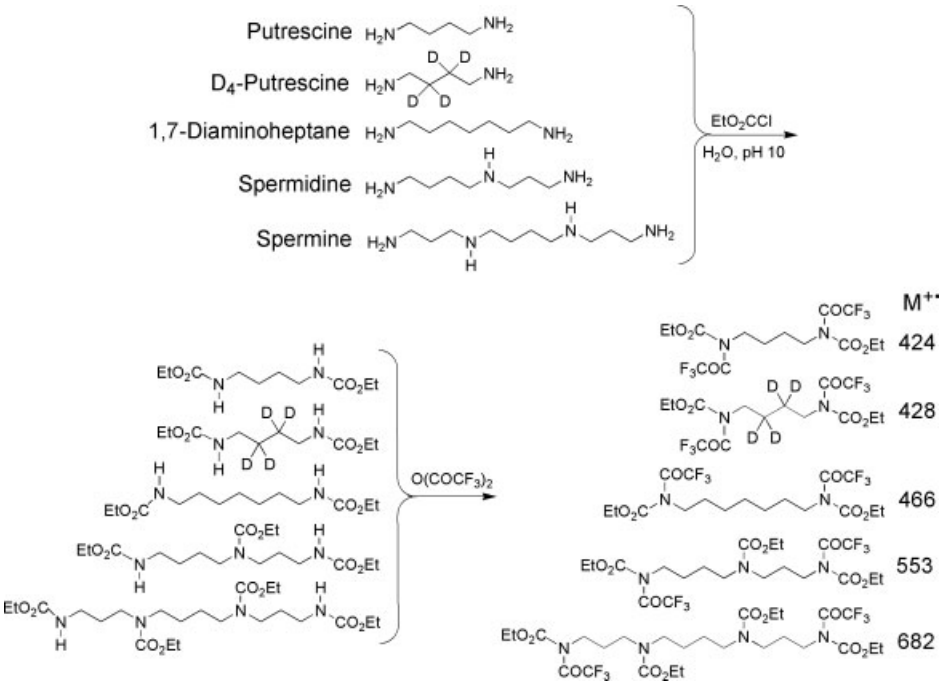


Figure 1. Polyamines studied and their derivatization.

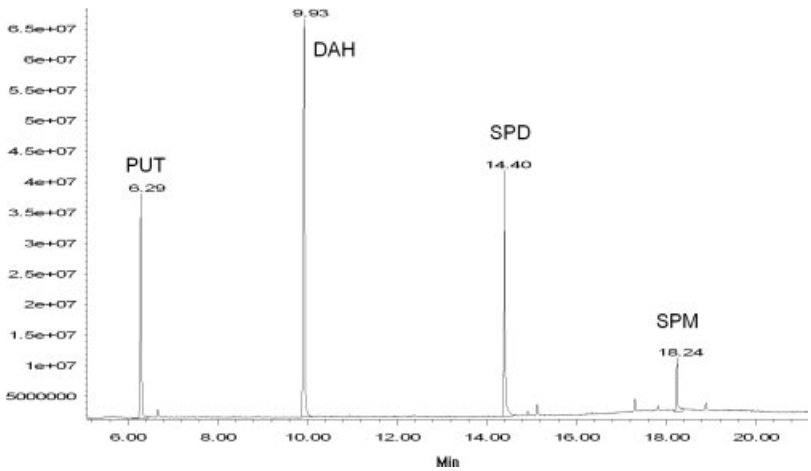


Figure 2. Total ion current chromatogram for the derivatives of the authentic amines in the study.

product of CDN Isotopes (Pointe-Claire, QC, Canada) having 98 atom % deuterium. A linear scan of the derivative confirmed this degree of labeling and no unlabeled PUT was detected. Other reagent grade chemicals were obtained locally. The postmortem brain tissue samples were acquired from the Quebec Suicide Brain Bank (www.douglasrecherche.qc.ca/suicide). Tissue donations to this unique resource and withdrawal of samples were ethically approved and are from males of French-Canadian origin, a homogeneous population having a well-documented founder history.

Preparation of polyamine standards

A stock solution of each polyamine standard was prepared at a final concentration of 10 mg/ml in 0.1 M HCl. Working standard solutions (at 100 ng, 1 µg, 10 µg and 1 mg/ml) were prepared by dilution of the stock solutions in 0.1 M HCl and stored below 4 °C.

Calibration

Pilot experiments were carried out to determine that the level of PUT-D_4 needed for PUT and the level of internal standard DAH needed to represent SPD and SPM was 10 ng/mg of tissue. Calibrating samples were prepared by the addition of 500 ng of PUT-D_4 and 500 ng of DAH internal standards to six 0.5 ml aliquots of 0.1 M HCl. To these were added increasing amounts of PUT, SPD and SPM. After dilution to 1 ml with 0.1 M HCl, the concentrations were 1 ng, 10 ng, 100 ng, 1 µg, 10 µg and 100 µg/ml, respectively. These samples were analyzed as described below. The limit of detection and linearity of response were determined. Tissue concentrations of the three polyamines were then calculated by interpolation on the linear portions of the calibration curves.

We used water as the matrix as we were unable to obtain the large mass of brain tissue that would have been required to formulate the calibrating samples in a brain tissue matrix.

Journal of MASS SPECTROMETRY

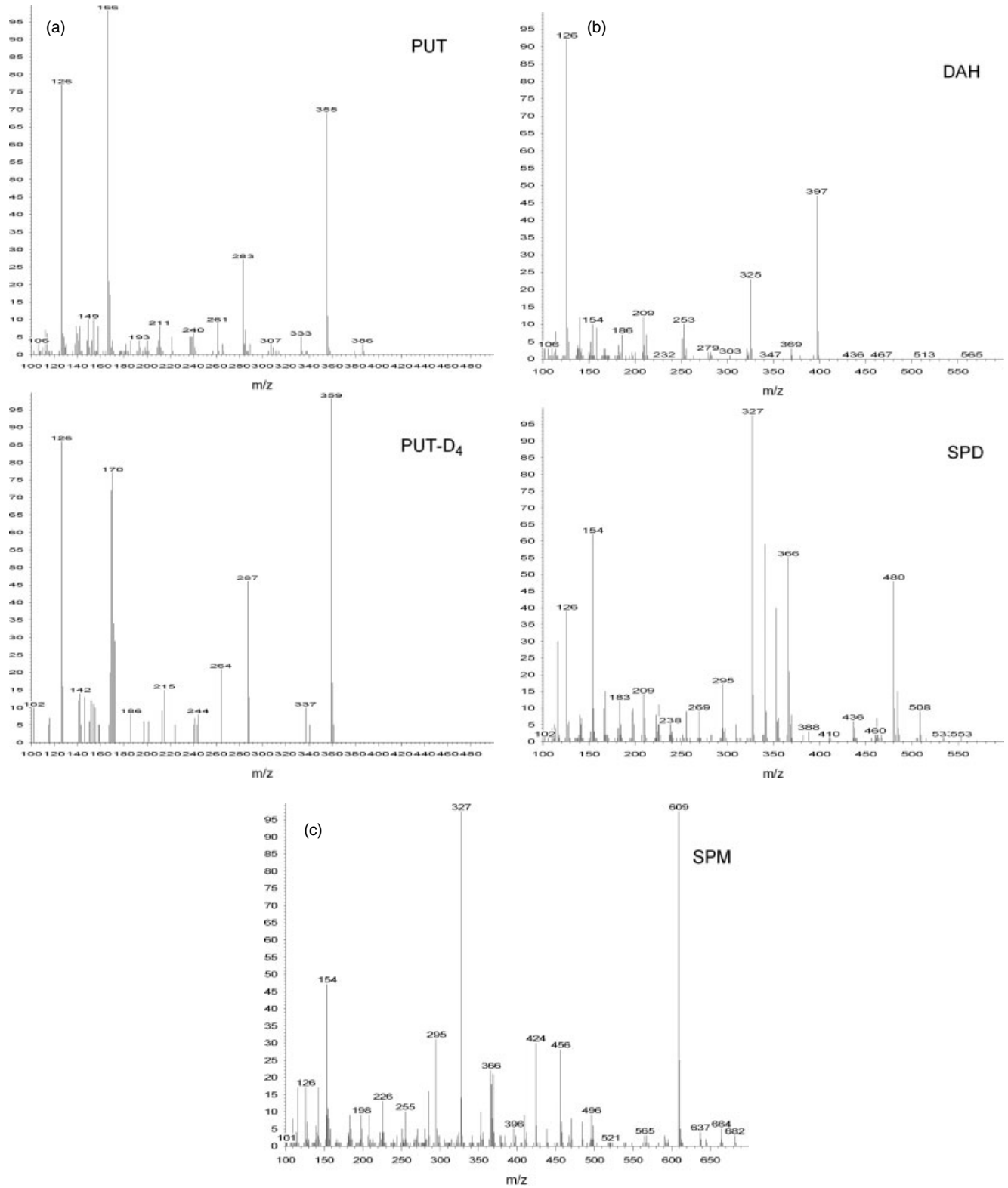


Figure 3. Full scan EI mass spectra of the derivatized amines. The *m/z* 327 was not selected for monitoring as it was found to be a common interfering ion.

Brain sample preparation and polyamine extraction

To approximately 100 mg of thawed tissue, PUT-D_4 and DAH internal standards were added at 10 ng per mg of tissue. The tissue samples were homogenized on ice with 10 volumes of 10% NaCl solution made pH 1 with HCl and then centrifuged at 12 000 × *g* for 15 min. The supernatant containing the soluble polyamines was collected and the pellet was re-suspended in 10 volumes of pH 1 saline solution and put through the homogenization process again. The combined supernatants were adjusted in volume to

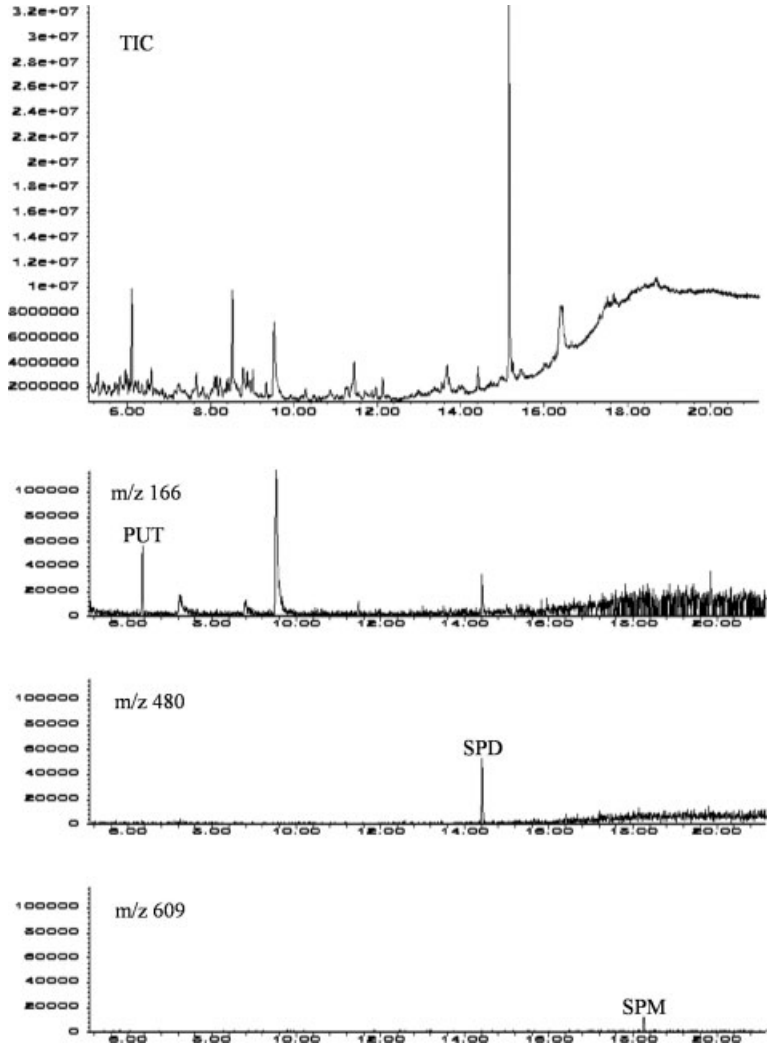


Figure 4. TIC and extracted ion chromatograms for a patient sample that was not spiked with internal standards or polyamines. Endogenous levels are apparent.

2 ml with pH 1 saline solution. The resulting sample was extracted with 3 ml of diethyl ether with vortexing for 10 min. The emulsion may be separated by centrifugation at 12 000 × *g* for 20 min. The ether layer that contains lipids, carbohydrates and other potential contaminants was discarded. The ether extraction was repeated and the aqueous phases were stored at −80 °C for subsequent derivatization.

Polyamine derivatization with ECF and TFAA

A scheme outlining the derivatization steps for these amines is illustrated in Fig. 1. A 0.5 ml aliquot of a polyamine standard solution or polyamine brain extract was adjusted to pH 10 ± 1 with 5 M NaOH. To carry out the *N*-ethoxycarbonylation of the amines, 1 ml of diethyl ether containing 50 µl of ECF was added to the sample solution. The reaction mixture was shaken at room temperature for 30 min, by which time CO_2 evolution has ceased, and then centrifuged at 1200 × *g* for 5 min. The ether layer containing the polyamine *N*-ethoxylcarbonyl (N-EOC) derivatives was transferred to a separate glass vial. This derivatization reaction is repeated by reextracting the aqueous phase with 1 ml of diethyl ether containing 50 µl ECF. The addition of further derivatization repeats was found not to increase recoveries, and analysis of these repeats produced samples that on GC-MS analysis contained no detectable polyamines.

The ether layers from the two extractions are combined and evaporated to dryness under a dry nitrogen stream. The dried N-EOC polyamine derivatives were taken up in 100 µl of ethyl acetate to which 200 µl of TFAA was added. The mixture in sealed vials was placed on a 75 °C heating block for 1 h to complete the trifluoroacetylation reaction. It was found that continued heating for more than 1 h was without effect. The mixture was then evaporated to complete dryness under a dry nitrogen stream. The derivatives were reconstituted in 200 µl of ethyl acetate and 2 µl aliquots were injected for GC-MS analysis in triplicate.

The yields of the fully N-EOC-N-TFA derivatized SPD and SPM which contain sterically hindered secondary amines were optimal with the conditions employed. We found no evidence of incompletely derivatized SPD or SPM in these analyses. The time required for the preparation of a small number of samples in parallel is about 6 hr.

Journal of MASS SPECTROMETRY

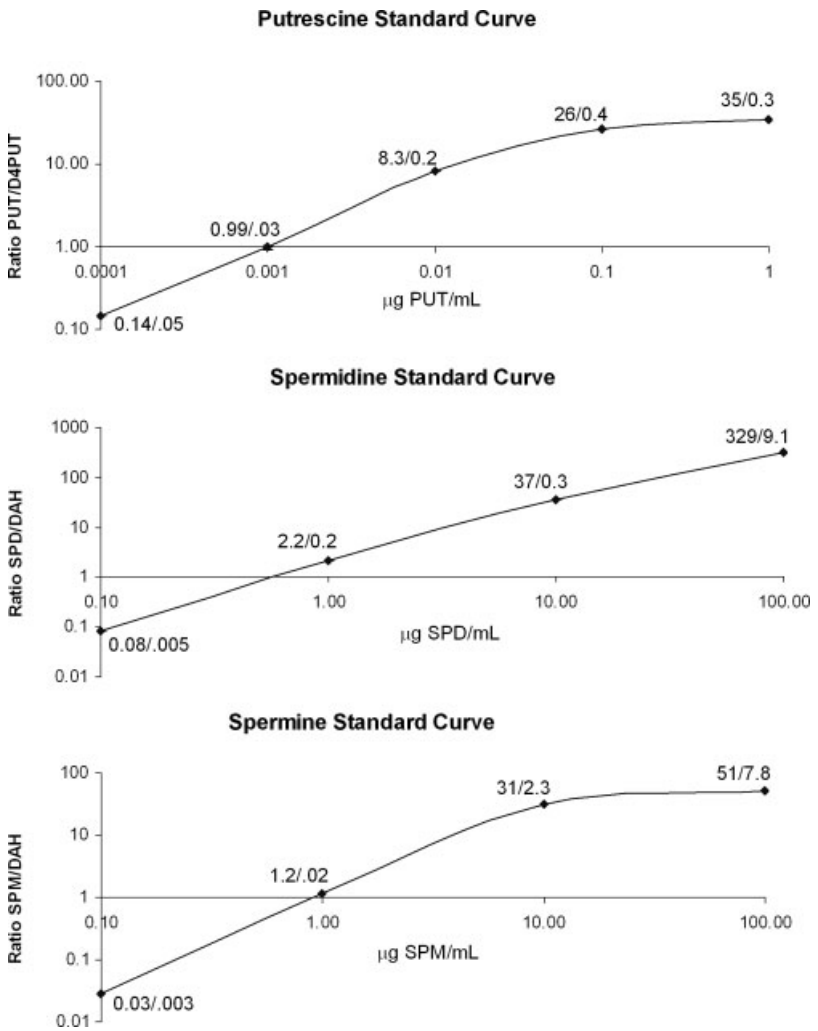


Figure 5. Calibrating curves in water for the three polyamines quantitated in this study. The weights of analytes per ml of calibrating sample is plotted *versus* the ratios of the intensities measured for the analytes and standards. Data points are annotated with the means/standard deviations for three sample injections. The physiological ranges are in the relatively linear portions of the plots.

Instrumental conditions

An Agilent bench-top HP6890/MSD5973N Chemstation system (Agilent Technologies. Inc. Santa Clara, CA, USA) was employed for this work. All mass spectra were acquired in EI for all measurements in full scan and SIM modes. Helium carrier gas was set to a column head pressure of 8.5 psi at a flow rate of 1.0 ml/min. Sample aliquots of 2 µl were injected in splitless mode with a 5-min solvent delay. GC analyses were with a HP-5MS capillary column (25 m, 0.25 mm i.d. and 0.25 µm thickness) programmed from 140 °C to 210 °C at 8 °C/min followed by a 2-min hold, then to 300 °C at 20 °C/min, followed by a 4-min hold. A final temperature increase to 320 °C at 20 °C/min was held as bake out for 4 min. MS conditions were the following: source 200 °C, quadrupole 150 °C, interface 250 °C, injector 260 °C, electron energy 70 eV and the typical source pressure was 1.8×10^{-5} torr. Full scans were over the mass range *m/z* 10–700 at 2.0 s/scan.

A total of 14 ions from four ion groups were used to monitor PUT, PUT-D_4, DAH, SPD and SPM. SIM and full scan acquisitions were started at 5.0 min and ended at 25.08 min. The first ion group consisted of six ions with *m/z* 166, 355 and 424 for PUT and *m/z* 170, 359 and 428 for PUT-D_4 running for 5–8 min with a dwell time of 100 ms. The second ion group consisted of two ions, *m/z* 397 and 466 for DAH, running from 8 to 13 min with a dwell time of 200 ms. The third ion group was *m/z* 295, 480 and 553 for SPD running from 13 to 16 min with 200 ms dwell time and the last ion group, *m/z* 424, 609 and 682 for SPM, started at 16 min and ended at 25 min with dwell time of 200 ms. While all ions in each group were monitored for peak verification, the ions used for quantification were those in italics above (Table 1).

Results and Discussion

The total ion current (TIC) chromatogram from a full scan GC-MS analyses of the authentic polyamine derivatives is shown in Fig. 2. The large range of volatilities of these derivatives necessitate a relatively long chromatographic run. Figure 3 shows the EI spectra of the derivatives of the authentic amines. While the molecular radical cations were visible in all cases, they were not intense enough to be useful in SIM as quantitating or confirming ions. The TIC and extracted ion chromatograms of a patient sample (Fig. 4) revealed many elutions due to column bleed and unidentified compounds. Endogenous concentrations of polyamines can be detected in the extracted ion chromatograms of this sample that

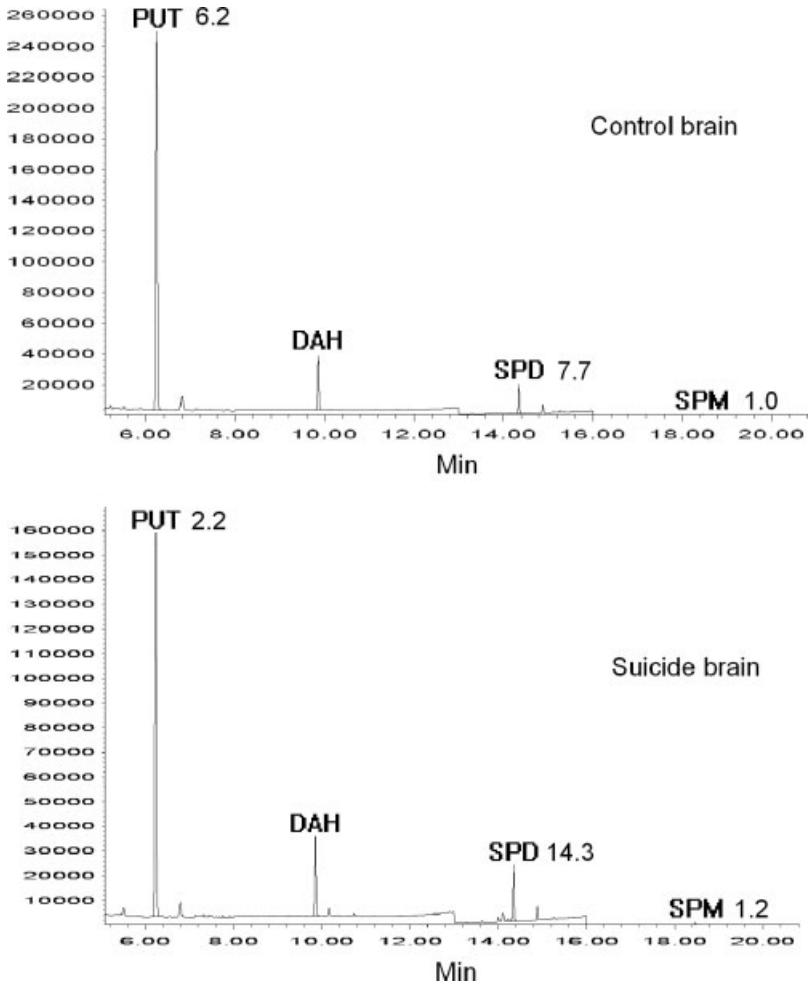
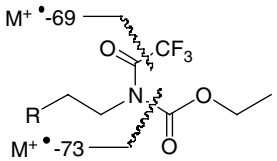


Figure 6. Composite selected ion chromatograms for the internal standards and the three major polyamines isolated from the cortex of a normal brain and one from a suicide victim. The polyamine concentrations are given as annotations to each polyamine peak and are expressed as ng/mg wet tissue. Alterations in the latter with respect to the former are statistically significant.

Table 1. Fragment ions monitored in selected ion monitoring (SIM)

Amine	$M^{+\bullet}$	$M^{+\bullet} - 69$	$M^{+\bullet} - 73$	$M^{+\bullet} - 258$
PUT	424	*355*		166
PUT-D_4	428	*359*		170
DAH	466	*397*		
SPD	553		*480*	295
SPM	682		*609*	424

PUT, putrescine; PUT-D_4, [2,2,3,3-$^2H_4$]-1,4-diaminobutane; SPD, spermidine; SPM, spermine.
Italicized masses were used for quantitating purposes in SIM, others for identity verification.



Structures of fragment ions monitored for quantitative SIM purposes. R is the balance of the molecular structures for PUT, PUT-D_4, DAH, SPD and SPM.

was not spiked with internal standards or polyamines. Although fragmentation in EI tends to reduce sensitivity, relatively high-mass fragments were intense enough for quantitating purposes. Other techniques such as positive ion chemical ionization that yield much less fragmentation concentrate intensities in protonated molecular ions and have been shown to be useful for related compounds.[10] We estimated the lower limits of detection (LOD) from the analysis of tissue samples that had low but easily quantitated polyamine concentrations. The LOD is calculated then as the concentration that would correspond to polyamine peaks having a signal to noise ratio of 10.

Figure 5 shows the calibrating plots obtained and used in the quantitation of the three polyamines. Nonlinearity at high analyte concentrations is noted particularly for SPM. We were unable to determine whether this was related to electron multiplier saturation or incomplete derivatization. We discount the latter as we found no evidence with authentic SPD or SPM of incomplete derivatization. The only peak in the chromatogram that was not the expected SPM derivative was due to a persistent small impurity of SPD. In all three cases, the physiological ranges were completely within the relatively linear portion of the calibration plots (Table 2). Even though the recoveries were not excellent, they were usefully consistent sample-to-sample. Since

Journal of MASS SPECTROMETRY

Table 2. Linearity and recovery data for tissue and calibration samples

Polyamine	LOD (ng/g wet tissue)	Calibration range (ng/ml)	Linearity (r)*	Recovery (%) from water
Putrescine	1.0	$1-10^3$	0.997	78
Spermidine	10	10^2-10^5	0.999	64
Spermine	100	10^2-10^5	0.881	60

LOD, limit of detection, estimated for hypothetical peaks having a signal to noise ratio of 10 in tissue samples.
* r measured as linear correlation coefficients.

incompletely derivatized polyamines were not detected, we can only speculate that in the protein and cell-debris precipitation step, recoveries of these somewhat lipophillic compounds and their internal standards were reduced by coprecipitation. Samples were analyzed by triplicate injections as we could not prepare and analyze three aliquots of the same tissue due to restrictions on the quantities of tissue samples to which we were permitted access.

Figure 6 illustrates a comparison of typical SIM chromatograms obtained for a control brain sample and one taken from a suicide victim. The chromatographic peaks for PUT, SPD and SPM are annotated with their respective concentrations (ng/mg tissue). The concentration of PUT in the normal brain was determined to be 6.2 ng/mg wet tissue. This is comparable with an earlier reported value (174 pmol/mg protein) for rat hippocampus[10] that is equivalent to 1.53 ng/g hippocampal tissue, assuming 100 mg protein per gram of wet tissue. The values reported in Fig. 6 represent statistically significant differences and are representative of further results collected in an ongoing study that will be published elsewhere when complete. At this point, a basis for a cause *versus* effect relationship between polyamine increases and depression is not proven.

Traditional liquid–liquid extraction has many disadvantages such as matrix interference, intractable emulsion formation, requirement of hazardous solvents and the requirement of more complex isolation protocols. Compared with solid phase and organic solvent extraction methods we have tried, the method described here reduced solvent exchanges, drying steps and sample transfer manipulations. These not only increase the extraction efficiency and convenience but also reduce chances of sample loss or error. This is especially important for polyamine extraction from limited resources such as postmortem brain tissue. Since extraction of polyamines with 0.1 M HCl is relatively mild, it avoids strong oxidizing acids such as perchloric acid used in other methods[11] that may induce isomerization or *N*-oxidation of a large polyamine molecule such as SPM.

N-Ethyloxycarbonylation of polyamines with ECF to form urethanes occurs in the aqueous layer at pH 10, and these are rapidly transferred into the top ether layer. At pH < 9, protonation of the polyamines appears to prevent complete derivatization and at pH > 11, hydrolysis of the product urethanes appears to be favored. Spermine contains two secondary amine groups making it the most difficult polyamine to completely derivatize and quantify with this method.

We elected to use trifluoroacetylation of the urethane intermediates instead of pentafluoropropionylation[9] as the *N*-ethoxylcarbonyl-*N*-trifluoroacetyl derivatives have smaller molecular weights and showed improved peak shape in GC-MS and SIM compared to the pentafluoropropionyl derivatives. TFAA also has the advantage of producing acidic by-products that are more volatile.

Conclusions

Measuring polyamines directly in postmortem brain tissue is particularly important considering recent evidence implicating polyamines in mental disorders and suicide.[12] A method for extraction and determination of the three major polyamines from human postmortem brains has been successfully applied in the analysis of more than 200 postmortem human brain samples in our laboratory. The repeatability and calibration tests were satisfactory over the physiological range. Extraction with 0.1 M HCl allowed efficient extraction of all three major polyamines from postmortem brain tissue. It is a relatively simple, efficient and inexpensive method compared with earlier methods. The detection limits are 1.0 ng/g of tissue for PUT and 10 ng/g and 100 ng/g tissue for SPD and SPM, respectively.

Acknowledgements

This work was supported by Canadian Institutes for Health Research grants to Gustavo Turecki (MOP – 79253) and a Canadian Foundation for Innovation grant to Orval A. Mamer.

References

[1] L. M. Fiori, G. Turecki. Implication of the polyamine system in mental disorders. *Journal of Psychiatry & Neuroscience* **2008**, *33*, 102.
[2] R. A. Casero Jr, L. J. Marton. Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. *Nature Reviews, Drug Discovery* **2007**, *6*, 373.
[3] A. E. Pegg. Spermidine/spermine-N(1)-acetyltransferase: a key metabolic regulator. *American Journal of Physiology, Endocrinology and Metabolism* **2008**, *294*, E995.
[4] Y. Maeno, F. Takabe, H. Inoue, M. Iwasa. A study on the vital reaction in wounded skin: simultaneous determination of histamine and polyamines in injured rat skin by high-performance liquid chromatography. *Forensic Science International* **1990**, *46*, 255.
[5] J. W. Suh, S. H. Lee, B. C. Chung, J. Park. Urinary polyamine evaluation for effective diagnosis of various cancers. *Journal of Chromatography, B: Biomedical Sciences and Applications* **1997**, *688*, 179.
[6] G. M. Gilad, V. H. Gilad, M. F. Casanova, R. A. Casero Jr. Polyamines and their metabolizing enzymes in human frontal cortex and hippocampus: preliminary measurements in affective disorders. *Biological Psychiatry* **1995**, *38*, 227.
[7] Y. Qiu, M. Su, Y. Liu, M. Chen, J. Gu, J. Zhang, W. Jia. Application of ethyl chloroformate derivatization for gas chromatography-mass spectrometry based metabonomic profiling. *Analytica Chimica Acta* **2007**, *583*, 277.
[8] J. O. Fernandes, M. A. Ferreira. Combined ion-pair extraction and gas chromatography-mass spectrometry for the simultaneous determination of diamines, polyamines and aromatic amines in Port wine and grape juice. *Journal of Chromatography A* **2000**, *886*, 183.
[9] M. H. Choi, K. R. Kim, B. C. Chung. Determination of hair polyamines as N-ethoxycarbonyl-N-pentafluoropropionyl derivatives by gas chromatography-mass spectrometry. *Journal of Chromatography A* **2000**, *897*, 295.
[10] P. L. Wood, M. A. Khan, J. R. Moskal. Neurochemical analysis of amino acids, polyamines and carboxylic acids: GC-MS quantitation of tBDMS derivatives using ammonia positive chemical ionization. *Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences* **2006**, *831*, 313.

[11] F. Custódioa, E. Tavaresa, M. Abreu Glória. Extraction of bioactive amines from grated Parmesan cheese using acid, alkaline and organic solvents. *Journal of Food Composition and Analysis* **2007**, *20*, 280.

[12] A. Sequeira, F. G. Gwadry, J. M. Ffrench-Mullen, L. Canetti, Y. Gingras, R. A. Casero Jr, G. Rouleau, C. Benkelfat, G. Turecki. Implication of SSAT by gene expression and genetic variation in suicide and major depression. *Archives of General Psychiatry* **2006**, *63*, 35.