ISSN-1996-918X

Pak. J. Anal. Environ. Chem. Vol. 19, No. 1 (2018) 28 – 35

http://doi.org/10.21743/pjaec/2018.06.02

Capillary Gas Chromatographic Determination of Gamma Aminobutyric acid and Putrescine in Cerebrospinal Fluid using Trifluoroacetylacetone as Derivatizing Reagent

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Received 28 November 2017, Revised 16 March 2018, Accepted 20 March 2018

Abstract

In present work, a new capillary gas chromatographic procedure was established and validated for the determination of gamma aminobutyric acid (GABA) and putrescine (Put) using trifluoroacetylacetone (FAA) as derivatizing reagent from Cerebrospinal Fluid (CSF) samples prior to their gas chromatographic- flame ionization detector (GC-FID) analysis. GABA, Put, cadaverine (Cad) and tyramine (TY) as imitative of FAA extracted from the column HP-5 (30 m x 0.32 mm i.d) at temperature 110 ºC for 1 min, tracked by heating rate 25 ºC to 260 ºC /min. The detection was carried out by FID with segregate ratio 10:1, *v/v* with whole run time 10 min. The proposed method showed linear calibration range between 2.5-50 µg/mL with low limit of detection 1.0 - 2.5 µg/mL analogous to 0.1 ng to 0.25 ng for selected Put, Cad, GABA, and TY. The method based on the pre-concentration was used for the determination of GABA and Put from CSF of human being and amounts found were 0.25- 0.56 µg/mL and 0.16 - 0.41 µg/mL with relative standard deviation (RSD) within 0.8 - 1.1 and 1.1 - 1.5 %, respectively. Many of amino-acids tested, separated completely and did not variate the determinations of GABA and Put.

Keywords: Trifluoroacetylacetone, Gamma aminobutyric acid, Putrescine, Amino-acids, Cerebrospinal Fluid, GC-FID.

Introduction

Gamma-aminobutyric acid (4-aminobutyric acid) (GABA) and putrescine (1,4-diaminobutane) (Put) are considered as an important compounds available in biological system [1]. GABA is inhibitory neurotransmitter and usually reduce the excess impulses, pain, anxiety, nervousness and addiction [1,2]. GABA is an amino acid which doesn't cross the brain barrier cell and is synthesized within the brain from glutamate by the enzyme glutamic acid decarboxylate [3,4]. While, Put and cadaverine (1,5-diaminopentane) (Cad) are positively charged aliphatic amines and found in living species [5]. These species plays a vital role and help the cells to produce or participate in an optimal manner. The concentration of Put is increased in biological fluids in cancer patients [5,6]. Up till now various analytical techniques including magnetic resonance spectroscopy [7-9], electrochemical sensor [10, 11], spectro-fluorimetry [12], spectrophotometry [13], radio receptor assay [14], capillary electrophoresis (CE) [15-17], liquid chromatography (LC) [18-22] and

gas chromatography (GC), thin layer chromatography [23-28] have been applied for the determination of the GABA, Put and Cad from biological fluids [5, 29-37]. Since, majority of these techniques are dreary, costly and time consuming with the consequent risk of environmental pollution. Literature survey indicated that comparatively Capillary GC due to its low cost, high sensitivity, easy operation, high resolution, trouble-free procedure for the extraction of organic compounds is renowned as one of the most robust technique [24, -27]. GC procedures for GABA are based on the derivatization with heptafluorobutyric anhydride [23, 25], and isobutylchloroformate [24] with mass spectrometric or electrons capture detection systems. Ethyl chloroformate (ECF) has extremity used for the GC determination of amino acids [38-42]. The use of acid anhydrides has some damaging effects on the GC column [28]. GC with flame ionization detection (FID) is frequently available and derivatization with suitable reagent enhances the sensitivity remarkably. The volatilization of the derivative also decreases the number of interferences. Acetyl group of trifluoroacetylacetone (FAA) might react with primary amino group to create Schiff base and extended carbon chain with CF_3 group could enhance the volatility of the derivative. FAA was reported as derivatizing reagent designed for GC determination of Put and Cad [6,43] and phenylpropanolamine [44].

The present work examines the use of FAA for the first time for the determination of GABA together with Put from human CSF using GC combined with FID system. The work also proposes FAA as a reacting agent for GC elution and extraction of amino acids and amines. Experimental conditions are optimized for the derivatization (pH, reaction time, temperature and amount of derivatizing reagent added), elution and extraction of the compounds. The procedure reports linearity, limits of detection (LOD), limits of quantitation (LOQ), repeatability (inter and intra day), accuracy and recovery of the method. Many of amines and amino-acids present together, eluted and separated completely.

Experimental

Material

Putrescine dihydrochloride, cadaverine dihydrochloride (Sigma, St. Louis, USA), gamma aminobutyric acid (GABA), tyramine (TY) and methanol (E-Merck, Darmstadt, Germany), trifluoroacetylacetone (Fluka, Bucks, Switzerland) were used. HCl (37%), KCl, CH_3COOH, NaCOOH, NH_4COOH, $NaHCO_3$, Na_2CO_3, NH_4Cl and NH_3 (40 %) were from E-Merck (Darmstadt, Germany). Glycine, valanine, lycine, L-aspartic acid, L-methinion, L- arginine and histadine were procured from Sigma (St. Louis, MO, USA). The stock solutions containing (1000 µg/mL) of GABA, TY, Put and Cad were prepared by dissolving appropriate amount of gamma aminobutyric acid, tyramine, putrescine dihydrochloride and cadaverine dihydrogen chloride in CH_3OH:H_2O (1:1, *v/v*) as well as volume was made to 25 mL. The stock solutions were kept at 4 °C and were diluted as required by means of CH_3OH:H_2O (1:1, v/v) on the same day. Buffer solutions in the pH series 1 – 10 at unit time were organized from HCl (0.1 M) - KCl (1 M) (pH 1 and 2), CH_3COOH (1 M) – NaCOOH (1 M) (pH 3 to 7), $NaHCO_3$ (1 M)- Na_2CO_3 (saturated) (pH 8 and 9), and NH_4Cl (1 M) –NH_3solution (1 M) (pH 10). Six CSF samples five from male and one female within the age of 23-44 years were gathered from neurosurgical zone of Liaquat University of Medical and Health Sciences Hospital, Jamshoro, during operation of ventrico-peritional shunt of the patients tormenting from Meningitis, brainy malaria and brain tumor. A portion of the CSF samples was collected with verbal consent and authorization of duty doctor and the helper of the patient.

Equipments

The pH measurement was done with an Orion 420 A, pH meter (Orion Research lnc. Boston, USA) with united glass electrode and reference internal electrodes. pH meter was calibrated with standard buffer pH 7 and check at pH 4 and 9. Gas chromatographic learning were carried out on Agilent model 6890-network GC

system (Agilent Technology lnc. USA), split/splitless injector functioned with split-mode, flame ionization detection (FID), hydrogen generator (Parker Balston Analytical Gas system, Parker Hannifin Havorhill, MA, USA) and pure nitrogen (British Oxygen Company, Karachi). The computer through Chemstation software controlled the gas chromatograph. A capillary column HP-5 (30 m x 0.32 mm i.d) by a layer thickness of 0.25 µm (J & W Scientific Corporation, USA) was utilized thought the learning.

GC-FID Procedure

The solution (1 - 2 mL) having Put (0.4 - 7.6 µg), Cad (0.4 -7.6 µg), GABA (0.4 -10 µg) and TY (1 – 10 µg) was added 2 mL of FAA (2 % in methanol-water 1:1, *v/v*) and 2 mL of sodium acetate buffer pH 7. The mixture was warmed at 70 ºC for 10 min and was cooled to room temperature, chloroform (1 mL) was put in/ inserted/included and contents were merged well. The layers were permitted to divide and organic layer was accumulated. The extraction was repetitive with chloroform (1 mL). The combined extract was dispersed under nitrogen atmosphere and rest material liquefied in ethanol (0.2 mL). The solution (1 µL) was injected along with compounds eluted from GC column HP-5 (30 m x 0.32 mm i.d) at an initial column temperature of 110 ºC C for 1 min, tracked by programmed heating rate of 25ºC onward to 260ºC/min with whole run time 10 min. Injection port and detector temperature were retained at 280 and 300 ºC, respectively. The nitrogen as carrier run rate was 4.5 mL/min. The split ratio was 10:1, *v/v* and nitrogen 45mL/min was used as make up gas. The flow rates of hydrogen and air for FID were maintained at 40 and 450 mL/min. Regular peak height (n=4) was calculated for the individual component.

Determination of GABA as well as Put from CSF samples

Human cerebrospinal fluids (CSF) (2 mL) were added to methanol (2 mL) and centrifuged at 12000 g for 15 min. The supernatant was accumulated and the GC-FID method was tracked. The quantitation was carried out from calibration curve prepared from seven standard solutions.

Analysis of GABA and Put from CSF by standard addition method

Human cerebrospinal fluids (CSF) (2 mL) were added to methanol (2 mL) and centrifuged at 12000 g for 15 min.The supernatant was alienated in two equal portions and one portion was treated as “GC-FID method”. The subsequent part was mixed with solution containing GABA (5.0 µg) and Put (5.0 µg) followed by GC-FID analysis. The quantitation was passed out from linear calibration curve and increment in the response for GABA and Put with added amounts.

% Recovery of Cad and TA from CSF by standard addition method

Human cerebrospinal fluids (CSF) (2 mL) were added to methanol (2 mL) and centrifuged at 12000 g for 15 min. Following the deprote-inization, supernatant was alienated in two the same portions and one portion was treated as “GC-FID method”. While, the remaining part was added to the solution containing Cad (4.0 µg) and TA (10.0 µg) and GC-FID analysis was carried out. The % recovery was calculated from linear calibration curve and increment in the response for Cad and TA with added amounts.

Results and Discussion

GABA, Put, Cad in addition to TY including primary amino group reacted with FAA to create Schiff bases. The resulting Schiff bases could change into ketoamine tautomeric forms with the formation of cyclic structure due to intra hydrogen bonding with better thermal stability (Fig. 1), suitable for quantitative GC determination.

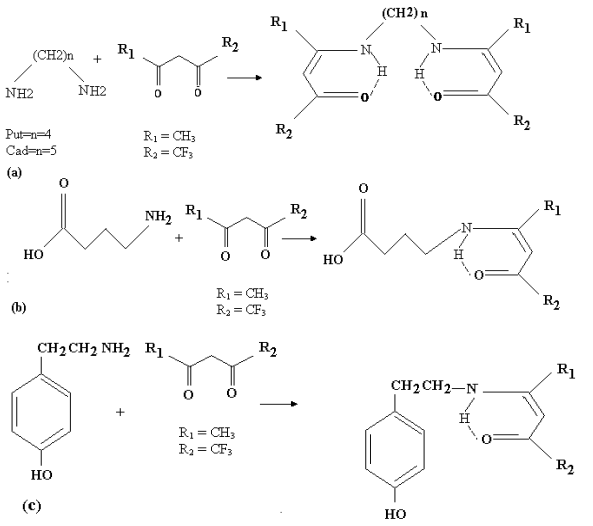

Figure 1. **Reaction of trifluoroacetylacetone (FAA) with (a) putrescine (Put) and cadaverine (Cad), (b) gamma aminobutyric acid (GABA) and (c) tyramine (TY)**

Optimization of derivatization and separation

The derivatives were examined on GC for their elution and the conditions for their formation were optimized. Every of the derivatives eluted from GC column gave a particular peak and alienated from the derivatizing reagent FAA. The effects of pH, derivatizing reagent amount and heating time on derivatization were examined. The solution (1 µL) was injected every time and situation which provided maximum response (peak height) was preferred. Different buffer solutions within pH 1 - 10, at unit interval were examined. The maximum response was obtained at pH 5 - 8 for GABA and Put, and pH 7 using sodium acetate buffer proved optimal for the extraction as FAA derivative, since at this pH electrostatic interactions increased (Fig. 2). Warming time at 70 ºC was changed between 5 - 25 min at an interval of 5 min and heating time of 10 min proved optimal and this probably increase the contact surface between the extraction agent and analytes. The amount of derivatizing reagent (2 % *v/v* in CH_3OH-H_2O 1:1, *v/v*) added was changed between 0.5 - 3.0 mL with an interval of 0.5 mL and same response was examined above the addition of 1 mL and 2 mL was selected. The solvents chloroform, 1,2-dichloroethane and methyl isobutyl ketone were studied for the extraction of derivatives and each solvent indicated a similar response, but chloroform was used for extraction and preconcentration of the derivatives. The derivatives did not show any change in response up to 24 hours and indicated high solution stability.

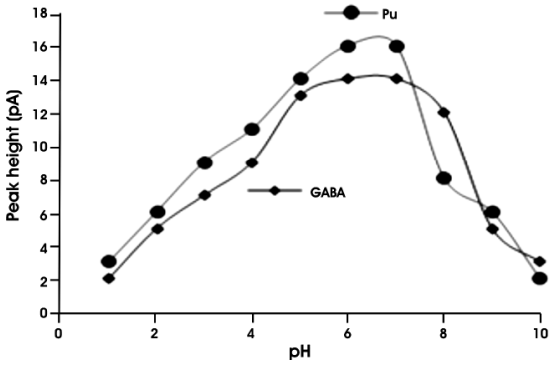

Figure 2. **Effect of pH on the extraction of FAA-GABA and FAA-Put derivatives**

The separation of Put, Cad, GABA, and TY as derivative of FAA was examined using different column temperatures and nitrogen run rates. The optimal separation was obtained at column temperature 110 ºC for 1 min, followed by programmed heating rate of 25 ºC/min up to - 260 ºC and nitrogen flow rate of 4.5 mL/min (Fig. 3).

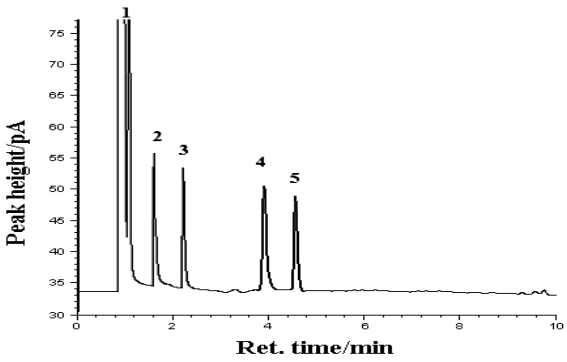

Figure 3. **GC separation of (1) solvent & reagent (2) Put (3) Cad, (4) GABA, and (5) TY as derivative of FAA**

The resolution factor (Rs) between two adjacent peaks was > 1.5. Other amino-acids containing primary amino group also react with FAA to form Schiff bases and elute from GC column. Glycine, valanine, lycine, L-asparatic acid, L- methinion, L-arginine and histadine when added together formed the derivatives with FAA and eluted from GC column and separated completely (Fig. 4). The peak recognition was

based on the time of retention of individual compound and spiking with standard solution.

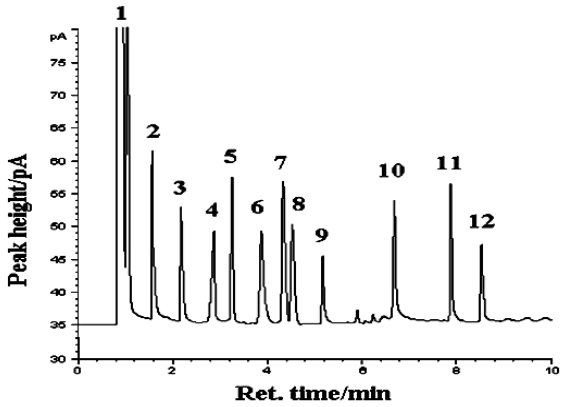

Figure 4. **GC separation of (1) solvent & FAA (2) Put (3) Cad (4) Gl (5) VAL (6) GABA (7) LYS (8) TY (9) L- Aspartic acid (10) L-methinion (11) L-Arginine (12) Histadine as derivative of FAA**

Validation and quantitation

The repeatability of the separation in expression of peak heights and retention times (n=5) was examined with an RSD of 1.0 - 1.5 % and 0.8 - 1.2 %, respectively. Linear calibration curve for GABA was obtained by plotting average peak height (n=4) against concentration and was obtained corresponding to 0.2 - 5.0 ng extending up to FID detector. The limit of detection calculated as signal to noise ratio (3:1) corresponded to 0.11 ng on the column. Similarly linear calibration curve for Put, Cad, TY were obtained with 0. 2 – 3.8 ng, 0.2 – 3.8 ng, and 0.5 – 5.0 ng with detection limits 0.08 ng, 0.1 ng and 0.26 ng, respectively. Limit of quantitation (LOQ) measured as three times the LOD was 0.33 ng, 0.24 ng, 0.3 ng and 0.78 ng for GABA, Put, Cad and TY reading up to FID, respectively (Table 1).

The standard deviations of intercept (Sa) and slope (Sb), and confidence limits of intercept (CLa) and slope (CLb) at 95% of linear calibration curves are summarized in Table 2. The procedure is based on preconcentration by the factor of 10. The detection limits in the original solution corresponded to 0.11 µg/mL GABA, 0.08 µg/mL Put and Cad and 0.26 µg/mL TY. The analysis of test mixture of GABA, Put, Cad and TY (n=4) to overcome the calibration range for each analyte specified a relative % error within ±3 %. The effects of amino-acids at double the concentration of GABA were examined following the GC-FID procedure and these did not affect the elution and determination of GABA and Pu with a relative error within ±4 %.

Table 1. **Quantitation GC data for FAA derivatives.**

S. No	Name of the compound	Calibration range µgmL^{-1}	Coefficient of determination (R^2)	Linear regression equation	Limit of detection (LOD) µgmL^{-1}	Limit of quantitation (LOQ) µgmL^{-1}	Sb	Sa	95% CLa	95% CLb
1	GABA	2.5-50.0	0.9982	Y = 0.4351x + .9418	1.1	2.5	0.039	0.748	±1.594	±0.055
2	Put	2.0-38.0	0.9992	Y = 0.6524x + .0667	0.8	2.0	0.134	0.886	±1.684	±0.194
3	Cad	2.0-38.0	0.9989	Y = 0.5821x + .0571	1.0	2.0	0.134	0.886	±1.684	±0.194
4	TY	5.0-50.0	0.9918	Y=0.3305x – 0.3452	2.5	5.0	0.134	0.886	±1.684	±0.194

Table 2. **Determination of GABA and Put from CSF.**

Sample No.	Disease	Age	sex	Amount of GABA found	Amount of Pu found
				µg/mL n=4	*µg/mL n=4*
1	Meningitis	23	male	0.28 (1.0)	0.16 (1.2)
2	Meningitis	25	male	0.34 (0.8)	0.19 (1.2)
3	Cerebral malaria	44	female	0.42 (0.9)	0.31 (1.0)
4	Brain tumor	31	male	0.45 (1.0)	0.23 (1.5)
5	Brain tumor	35	male	0.56 (1.1)	0.41 (1.0)
6	Cerebral malaria	38	male	0.25 (1.0)	0.18 (1.1)

Sample analysis

Six samples of CSF collected from the patients of meningitis, cerebral malaria and brain tumor were analyzed for contents of GABA and Pu. The GABA and Put are biological active compounds and are reported to be present in CSF at the concentration within 98.6 ± 33.9 ng/mL [45] and 4.4 ng/L [37], respectively. The amount of GABA and Put were found to be 0.25-0.56 µg/mL and 0.16-0.41 µg/mL with an RSD of 0.8 - 1.1 % and 1.1 -1.5 %, respectively (Fig. 5).

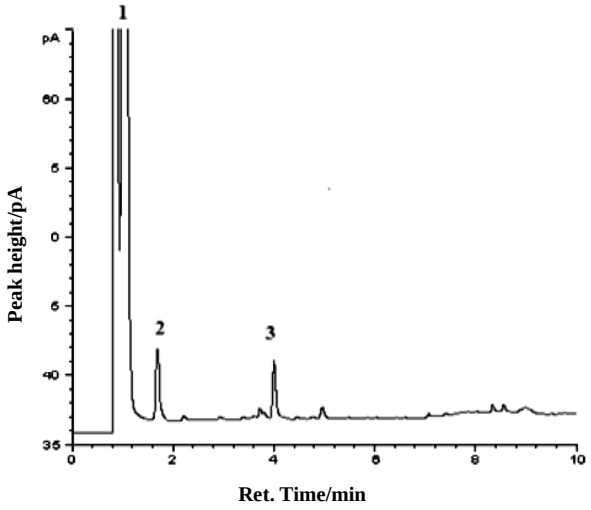

Figure 5. **GC response for (2) Put and (3) GABA from CSF sample**

The GABA and Put were also determined in CSF by standard addition method and amount was found to be 0.28 µg/mL GABA, 0.16 µg/mL Put and % recovery was calculated to 97.0 % and 97.8 %, respectively. The observed values for GABA and Put are higher than the reported values, may be because of different stages of the diseases of the patients [46, 47]. The compound Cad and TY were observed below the LOD in CSF samples. Deproteinized CSF was spiked with Cad and TY and responses obtained were compared with same concentration of Cad and TY extracted after derivatization from aqueous solution. The amounts of Cad and TY extracted from CSF were calculated to 97.5 % and 97.0 % with RSD of 1.8% and 2.2%, respectively (Fig. 6).

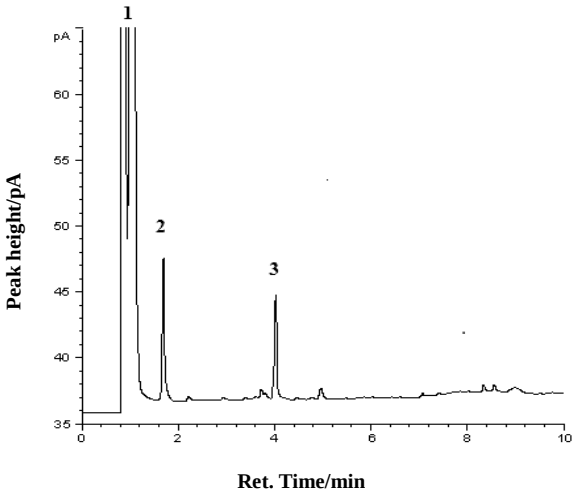

Figure 6. **GC response of (2) Put and (4) GABA from the CSF after spiking with 5.0 µgmL^{-1} of each**

Conclusion

A new capillary GC procedure has been developed for the determination / Quantitation of GABA and Pu from CSF samples of patient suffering from meningitis and brain tumor after derivatization with FAA with RSD within 0.5-1.5%. The detection limits were obtained with in 0.1 - 0.5ng reaching to the FID. The recovery of GABA and Put deproteinized CSF was calculated within 97.0-97.8%. A number of amino acids and amino compounds did not affect the determination.

Acknowledgement

Pakistan Science Foundation Islamabad is acknowledged for the Grant of Research Project S-SU /Chem (381) and Dr. Raja Riaz neurosurgeon for providing CSF samples. The authors also thank the Shaheed Benazir Bhutto University, Shaheed Benazirabad, Sindh, Pakistan for the financial support of this work.

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