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# Simple method for the quantitative analysis of endogenous folate catabolites *p*-aminobenzoylglutamate (pABG) and its acetamido (apABG) derivative in human serum and urine by liquid chromatography-tandem mass spectrometry

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**Abstract**

*Objective:* To develop a routine method for quantitative measurement of the folate catabolites *p*-aminobenzoylglutamate (pABG) and acetamidobenzoylglutamate (apABG) in serum and urine using liquid chromatography-tandem mass spectrometry (LC–MS/MS).
*Design and methods:* Urine, serum and aqueous standards were thawed. Two microliters of $d_3$-glutamic acid ($d_3$-Glu; 1 mmol/L) was added to 200 uL of specimen as internal standard. The samples were acidified with 4 uL 6 N HCL, and aliquots were precipitated with 2 volumes (412 uL) of acetonitrile. For urine specimens 30 volumes (6.18 mL) of acetonitrile was used. Samples were centrifuged at 1900 × *g* for 10 min and the supernatant (10 μL) injected into a Biorad CAT/MET analytical column fitted to the LC–MS/MS. Detection of the catabolites was by selective multiple ion monitoring (multiple SRM) of the respective transitions. Urine and serum samples were analysed in a group of healthy volunteers and in anonymous samples from patients being tested for PTH and urinary catecholamines.
*Results:* pABG and apABG eluted at 5.2 and 4.74 min, respectively while the $d_3$-glutamic acid eluted at around 7 min. Limit of quantitation (LOQ) for both catabolites was 10 nmol/L (which is equivalent to 33.3 fmol for a 10 μL injection). Limit of detection (LOD) was 1 nmol/L based on a signal to noise ratio of 5:1. A linear calibration curve was obtained from 10 to 100 nmol/L for serum specimens and from 10 to 200 μmol/L for urines. Imprecision for spiked serum samples ($n = 10$) was between 2.5 and 20% for apABG and 4.5 and 21% for pABG (at 10 and 100 nmol/L, respectively). Imprecision for spiked urine samples ($n = 10$) was between 2.9 and 4.0% for apABG and 6.0–12.7% for pABG. Recoveries were between 80 and 122% for serum samples and between 92 and 102% for urine specimens. Total folate catabolites in random urine samples from volunteers ($n = 5$) are 2.9 ± 2.3 umol/L (mean ± S.D.). This group also had total serum catabolites of 11.9 ± 7.6 nmol/L and serum folate of 35.3 ± 5.8 nmol/L. Serum from patients being tested for PTH ($n = 11$) had serum folate levels of 27.0 ± 10.4 nmol/L with total serum catabolites of 20.4 ± 23.8 nmol/L. Levels of serum folate and total catabolites in pregnant women ($n = 18$) were 33.9 ± 22.7 and 11.4 ± 8.7 nmol/L, respectively. Mean urinary folate catabolites in patients being tested for urinary catecholamines ($n = 19$) was 581.8 ± 368.4 nmol/L.
*Conclusion:* A simple, reliable and highly specific method by LC–MS/MS for detecting and quantifying the folate catabolites pABG and apABG was developed. This enables, for the first time, the routine clinical analysis of folate utilization in patients.


*Keywords:* pABG; apABG; Folate catabolite; Folate; Tandem mass spectrometry

## 1. Introduction

Folic acid is a water-soluble B vitamin that has received a lot of attention because of its purported role in the pathogenesis of cardiovascular disease [1–3], neural tube defects [4] and certain cancers [5]. Folate is important because it participates in a number of metabolic pathways as a cofactor involved in the transfer of one-carbon groups [6].

The amount of biologically active folate excreted by mammals is considerably less than that which is ingested. It has long been suggested that the major daily turnover for folate is via excretion of the catabolites *p*-aminobenzoylglutamate

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(pABG) and acetamidobenzoylglutamate (apABG) [7,8]. Thus, the obligatory requirement of folic acid, like with any other vitamin can be related to this catabolism. Certain conditions, such as pregnancy, periods of rapid growth, drug therapy regimes and a variety of clinical conditions, can increase folate catabolism and hence, its requirements.

The principal function of folate coenzymes, is to accept or donate one-carbon units in folate-requiring metabolic pathways. Cellular folates in their reduced form are conjugated to a polyglutamate chain, a mixture of unsubstituted polyglutamyl tetrahydrofolates and various substituted one-carbon forms of tetrahydrofolate (such as 10-formyl, 5,10-methylene, and 5-methyl) (Fig. 1).

The reduced forms of the vitamin, particularly the unsubstituted dihydro and tetrahydro forms, are chemically unstable and are easily split between the C-9 and N-10 bond to yield a substituted pteridine and *p*-aminobenzoylglutamate, which have no biological activity and are excreted in urine (Fig. 1) [9–11]. Substituting a carbon group at N-5 or N-10 decreases the tendency of the molecule to split. The substituted forms, however, are also susceptible to oxidative chemical rearrangements and, consequently, loss of activity [9]. The major folate catabolite in urine, however, consists of apABG whereas pABG exists in small amounts [8,11].

Early studies of folate catabolism in humans and laboratory animals have involved the administration of radiolabelled tracers, such as [$^{14}$C]pteroylmonoglutamate and [$^{3}$H]pteroylmonoglutamate, and the subsequent analysis of the excreted catabolites in urine. McNulty et al. [7] developed a reverse-phase high performance liquid chromatographic (HPLC) method for the quantitation of endogenous folate catabolites independent of in vivo administration of radiolabelled tracers. This method, like the earlier ones, required extensive sample preparation and cleanup to achieve detection and quantification of the urinary catabolites.

We demonstrate in these experiments, the development of a novel, versatile and simple method for the detection and quantification of the folate catabolites *p*-aminobenzoylglutamate and its

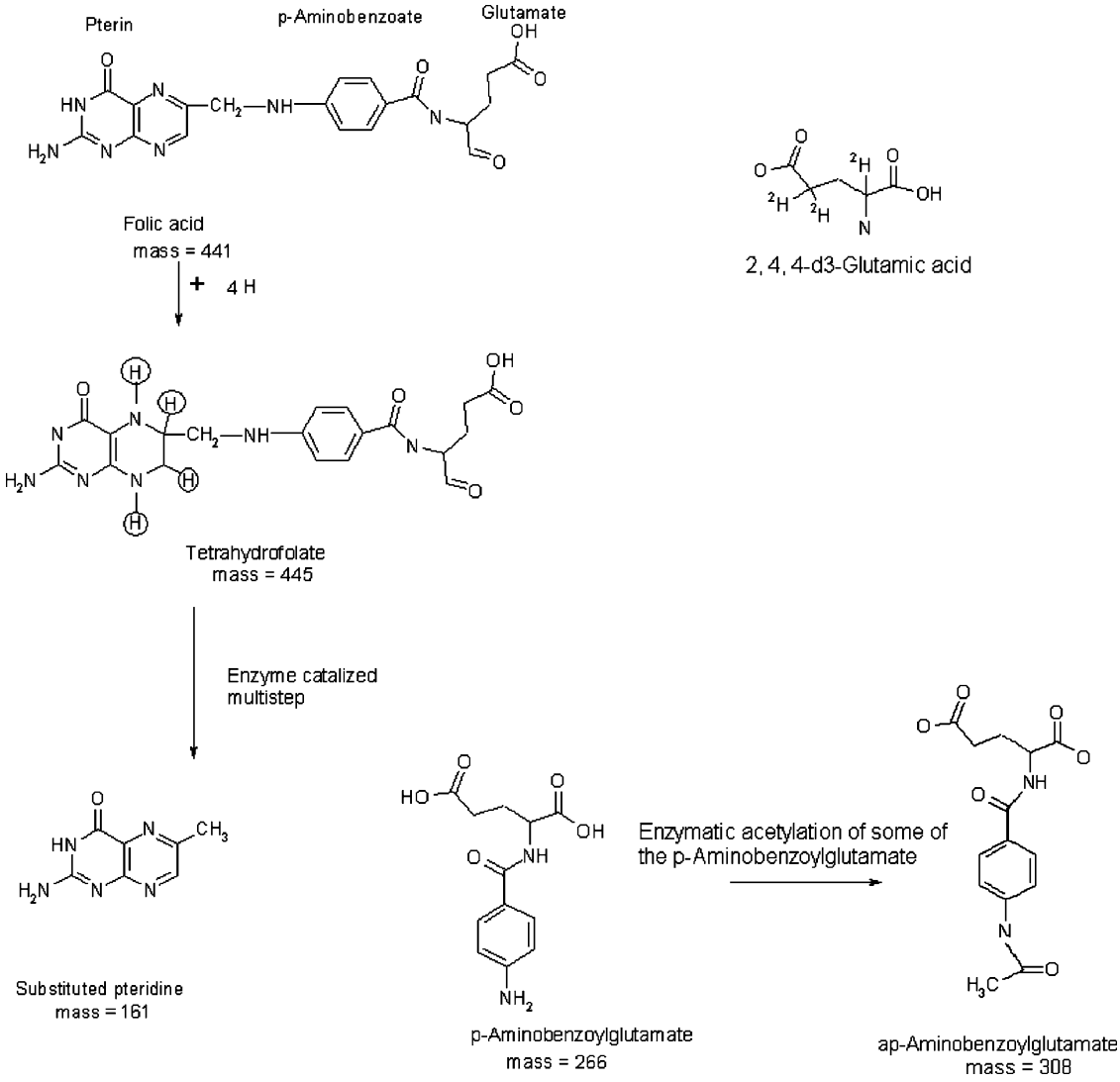


Fig. 1. Metabolism of folic acid in the body. Hydrogen moeity placement with the molecule is indicated by Ⓗ. Inserted in the figure is the structure of the internal standard used in the experiment.

acetamido derivative, acetamidobenzoylglutamate, in serum and urine using liquid chromatography-tandem mass spectrometry (LC–MS/MS). Sample preparation and cleanup is achieved in one step involving precipitation with acetonitrile. The resulting supernatant is injected into a normal phase Biorad CAT/MET analytical column fitted on an Agilent 1100 HPLC system coupled to a Sciex API 4000 quadrupole tandem mass spectrometer with an electrospray ionization source. Detection of the catabolites is by selective multiple ion monitoring (Multiple SRM) of the respective transitions.

## 2. Experimental

### 2.1. Materials and methods

Pure pABG Standard was obtained from Sigma–Aldrich (Oakville, ON, Canada). Acetamido derivative was prepared from the pABG by adding 140 uL of 50% acetic acid (VWR Canlab, Toronto, ON, Canada) in deionized water (Arctic Glacier, Regina, SK, Canada) (v/v) and 20 uL acetic anhydride (Sigma–Aldrich, Oakville, ON, Canada) to 10 mg of pABG. The reaction mixture was mixed vigorously and incubated in the dark at room temperature for 1 h. The remaining acetic acid was allowed to evaporate under a stream of nitrogen at room temparature. Stock solutions of both the pABG and the apABG were prepared in deionized water and contained 1.5 mmol/L each, respectively. Aqueous serial dilutions were made from each of the stocks at concentrations of 100, 50, 10 nmol/L and blank for serum and 200, 100, 50, 10 μmol/L and blank for urine specimens. These, together with the working stock solutions were stored at −20 °C.

$d_3$-Glutamic acid ($d_3$-Glu) (Fig. 1) was purchased from CDN isotopes (Pointe-Claire, QC, Canada) and used as internal standard. A working stock solution of 1 mmol/L was prepared in deionized water. HPLC grade acetonitrile (Anachemia Canada Inc., Lachine, QC, Canada), ammonium acetate (VWR canlab, Toronto, ON, Canada), formic acid (Fisher Scientific, Ottawa, ON, Canada) and deionised water were used to prepare the HPLC mobile phase.

### 2.2. Recovery experiments

Pooled serum (from patients that are being tested for thyroid hormones) and urine samples (from patients being tested for drugs of abuse) were spiked with pABG and apABG to required concentrations (100, 10 nmol/L, and blank for serum and 100, 10 μmol/L and blank for urine samples). Aqueous standards were prepared as described above. Two microliters of $d_3$-glutamic acid (1 mmol/L) was added to each 200 uL specimen as internal standard. Each sample was then acidified with 4 uL 6 N HCL (Fisher Scientific, Ottawa, ON, Canada) and vigorously vortexed. Precipitation/dilution (×3 for serum samples and ×30 for urines) was achieved by addition of 100% acetonitrile, vortexing and spinning at 1900 × *g* for 10 min. The resulting supernatant was then pipetted into a standard 96-well plate and placed on the Agilent 1100 well-plate autosampler.

### 2.3. Analysis of clinical samples

Blood and urine samples were collected after informed consent from a group of five healthy volunteers working in our lab. Blood was collected in a serum separator tube to yield serum, which was immediately analysed for serum folate on the Architect automated immunoanalyzer (Abbott Laboratories, Abbott Park, IL, USA). The rest of the sample was analysed for pABG and apABG within the hour as described above. Urine samples were collected in sterile urine containers, labelled, acidified to pH 3 with 6N HCL and analysed for pABG and apABG; also within an hour.

Serum samples ($n = 11$) collected at various collection centres, frozen within six hours and sent to our lab for PTH assay were analysed for pABG and apABG on the same day as PTH assay. A group of 18 serum samples from pregnant women was collected from a batch of serum samples sent to this lab for analysis of prenatal panel (rubella Ig G, syphilis, hepatitis B virus antigen and HIV antigen). Urine samples (24-h urine samples stored at 4 °C during collection) were collected from samples sent for urinary catecholamine assay in our lab ($n = 19$) after being acidified to pH 3 with 6N HCL. These urine samples were also analysed for pABG and apABG on the same day as received from the urinary catecholamine bench.

The results obtained were subjected to a modified Levene equal-variance test and subsequent two-tailed, two sample *t*-test using NCSS 2000/PASS 2000 Dawson Edition statistical software package (NCSS, Kaysville, UT, USA).

### 2.4. Instrumentation

Liquid chromatography-tandem mass spectrometry of the samples was done on an Agilent 1100 LC system, which includes a vacuum degasser, a binary pump, a well-plate auto-sampler and a heated column compartment. This system was coupled to an Applied Biosystems MDS Sciex API 4000 triple quadrupole tandem mass spectrometer. The chromatographic column consisted of a normal phase CAT/MET analytical column (catalog # 195-6033, Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase composition consisted of 2 mM aqueous ammonium acetate buffer with 0.1% formic acid (pH 3.0) and 100% acetonitrile as the organic phase. Chromatography consisted of an isocratic run of 50:50 buffer to organic phase. Flow rate was set at 300 μL/min, column temperature at 35 °C and an injection volume of 10 μL was used. A 15-s flush-port needle wash was incorporated into the procedure to prevent cross-contamination between injections.

Selective multiple ion monitoring (Multiple SRM) was done on the mass spectrometer using the Sciex Turbo ion spray source in positive ion mode. Resolution in quadrupole 1 (Q1) and quadrupole 3 (Q3) chambers were at unit mass. Other source parameters were as indicated in Table 1. The Multiple SRM mass transitions monitored were 309 > 162, 309 > 180, both for apABG and 267 > 120 for pABG. The dwell time for all the analytes was set at 500 ms. Run time for each LC injection was set at 10 min. Data was acquired and analyzed using Analyst software 1.4 (Applied Biosystems MDS Sciex).

Table 1
Source parameters in the MS/MS

| Instrument parameter | Value |
|---|---|
| Source temperature | 450 °C |
| Collision associated dissociation (CAD) gas | 6 psi |
| Curtain gas | 25 psi |
| Gas source 1 and 2 (GS 1 and GS 2) | 30 psi |
| Ion spray voltage | 5500 V |
| Declustering potential (DP) for both transitions 309 > 162 and 309 > 180 (apABG) | 38 V |
| Declustering potential (DP) for transition 267 > 120 (pABG) | 35 V |
| Declustering potential (DP) for transition 151 > 87 ($d_3$-glutamic acid) | 34 V |
| Electrode potential (EP) | 10 eV |
| Collision energy (CE) for transition 309 > 162 | 19 eV |
| Collision energy (CE) for transition 309 > 180 | 14 eV |
| Collision energy (CE) for transition 267 > 120 | 12 eV |
| Collision energy (CE) for transition 151 > 87 ($d_3$-glutamic acid) | 23 eV |

## 3. Results

Fig. 2a and b show spectral analysis of pABG and apABG and the proposed fragmentation chemistries for each. Mass spectral data were achieved through infusion of aqueous methanolic standards directly into the MS/MS. The respective ions and fragment ions were used to determine which SRMs would be monitored.

A five-point linear calibration curve was constructed for the aqueous standards of pABG and apABG. The chromatography of these aqueous standards showed complete separation of the analyte and the internal standard ($d_3$-glutamic acid). Spiked serum and urine samples showed chromatography consistent with that seen in the aqueous standards (Figs. 3 and 4). The acetylation process of pABG to apABG resulted in a 100% conversion within the 1-h incubation period as determined by the Q1 scan data before and after derivatization of pABG (data not shown).

### 3.1. Linearity

A four-point calibration curve for serum and five-point curve for urine were constructed for the standards. The dynamic range selected was based on both the limits of detection and the findings of Caudill et al. [15] in which the baseline concentrations of the pABG was around 24 nmol/L in non-pregnant women with folate supplementation of 450 μg/day of folic acid

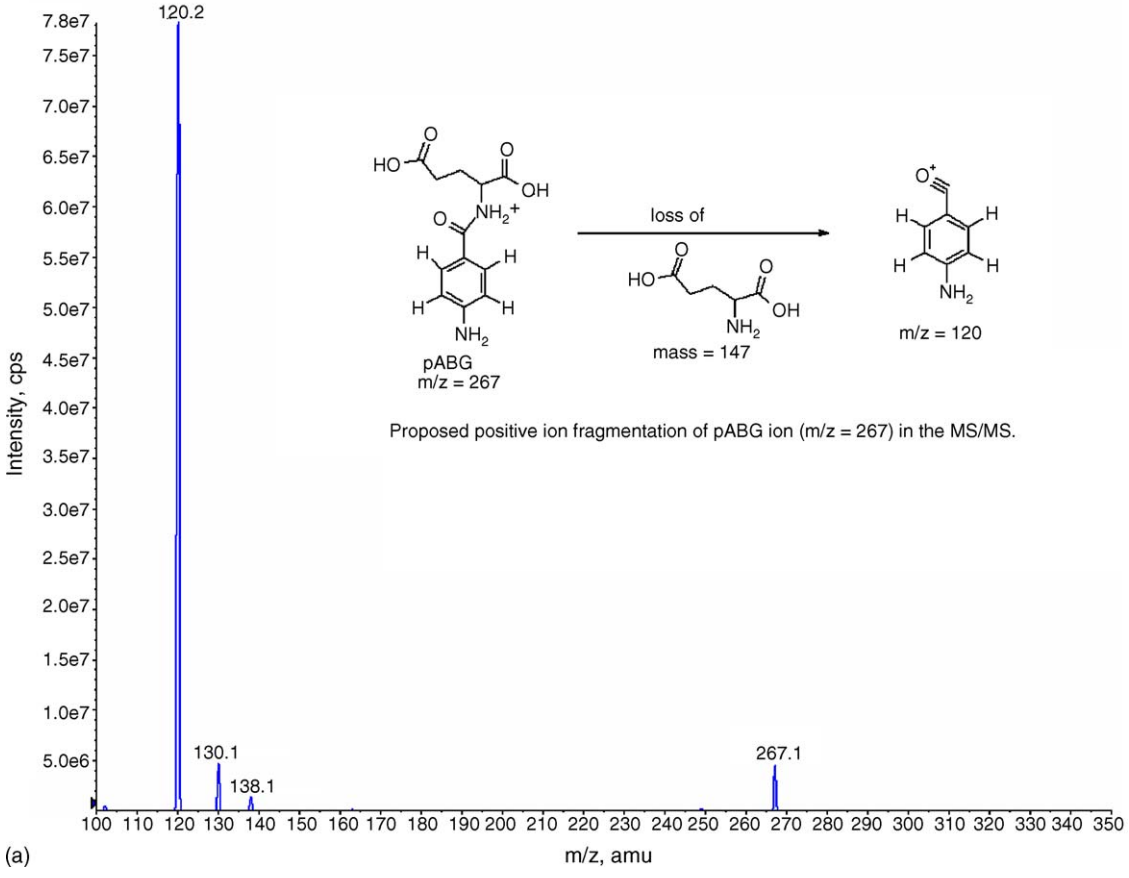


Fig. 2. (a) Product ion scan of $m/z = 267$ (pABG) (b) Product ion scan of apABG $m/z = 309$ (apABG).

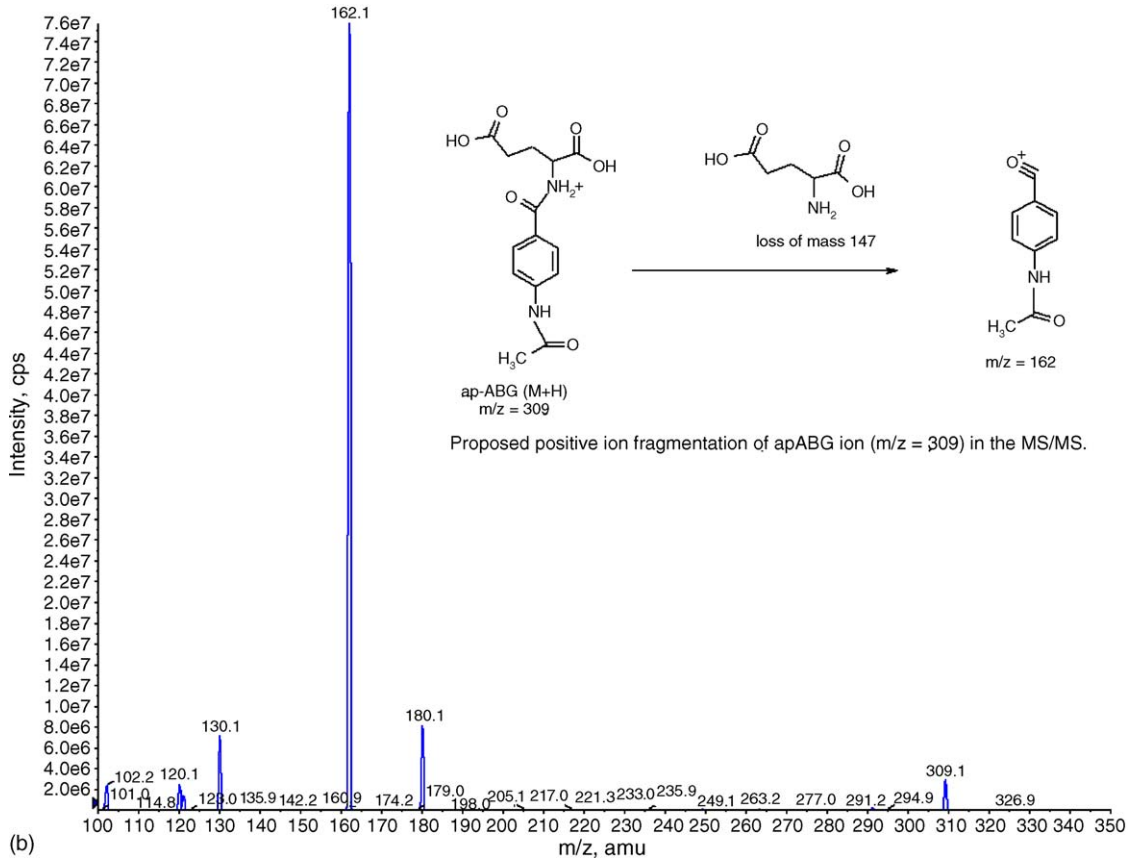


Fig. 2. (*Continued*)

and around 63 nmol/L in pregnant women with 850 μg/day of folic acid supplementation. The standard curve calibration points included a blank, 10, 50 and 100 nmol/L for serum and blank, 10, 50, 100 and 200 μmol/L for urine. 2 uL of 1 mmol/L $d_3$-Glu was added to 200 uL of specimen as an internal standard. Linear through zero regression (no weighting) equations were $y = 0.0111x$ ($r = 0.9998$) for apABG 309>162 transition, $y = 0.00131x$ ($r = 0.9999$) for apABG 309>180 transition and $y = 0.00194x$ ($r = 0.9998$) for the pABG 267 > 120 transition.

### 3.2. Precision and recovery

Intra-assay precision of apABG and pABG for serum and urine specimens was determined at two levels (10 and 100 nmol/L for serum and 10 and 100 μmol/L for urine). Pooled serum and urine samples from various patients (sent for various assays to our reference lab) were divided into ten different aliquots and spiked with apABG and pABG at the above two levels. The limit of detection (LOD) was 0.1 nmol/L in serum corresponding to a signal to noise ratio of 5:1. Limit of quantification (LOQ) was 1 nmol/L, which equals 33.3 femtomole (fmol) (absolute amount of sample). Table 2 shows the results of the precision and recovery assay.

Although all transitions showed good recovery, two transitions (267 > 120 at 100 nmol/L serum and 309 > 180 at 100 nmol/L serum) had higher CV values (>20%). These high CV values were contributed by two samples and may also be due to an unidentified co-eluting substance. While we did not exclude these results, we recommend that transition 309/162 be used as a specific marker of apABG.

### 3.3. Clinical samples

The mean age of the group for the lab volunteers was 40 ± 1 years (mean ± S.D.). Serum folate levels in this group was 35.3 ± 5.8 nmol/L. Total serum catabolites (pABG + apABG) were 11.9 ± 7.6 nmol/L (mean ± S.D.) with 75% of it being in the form of pABG and 25% in the form of apABG. Total urinary catabolites were 2.9 ± 2.3 μmol/L (mean ± S.D.) with pABG consisting of 27 and 73% made up of apABG.

The urinary catecholamines group ($n = 19$) had a mean age of 50 ± 17 years. Total urine catabolites in this group were 581.8 ± 368.4 nmol/L. apABG made up 76% while pABG consisted of 24%. The values and relative ratios of the urine catabolites seen here resemble that seen in the volunteers group despite the significant age difference in the means.

Mean age for the PTH group ($n = 11$) was 55 ± 17 years. Mean serum folate levels were 27.0 ± 10.4 nmol/L. Total serum catabolite levels in this group were 20.4 ± 23.8 nmol/L. 37% of the total folate catabolites was made up of apABG. The remaining 64% was made up of pABG.

Mean age for the pregnant group ($n = 18$) was 27 ± 6 years. Serum folate levels were 33.9 ± 22.7 nmol/L. Total serum

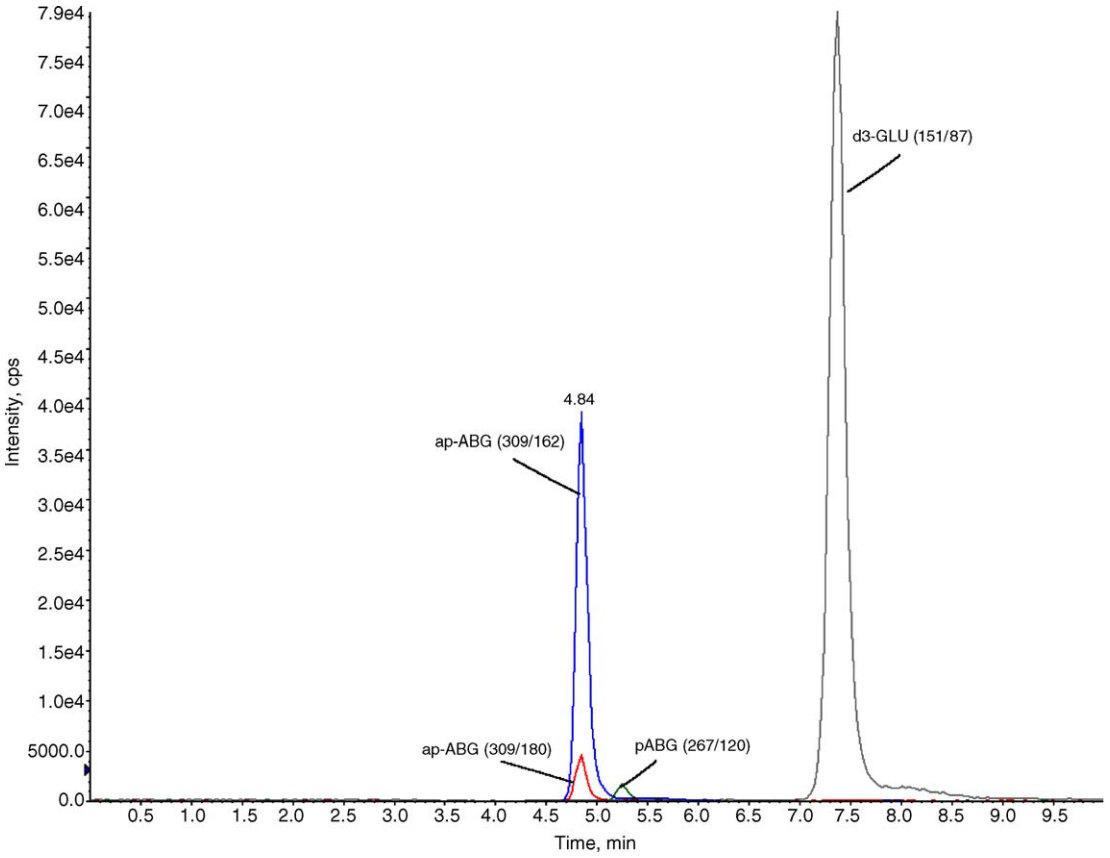


Fig. 3. LC–MS/MS–SRM of pABG, apABG and d3-Glu in 100 nmol/L spiked serum.

Table 2
Recovery and precision of spiked serum and urine samples

| Sample | Analyte | Transition | Concentration | Precision | Recovery (%) |
|---|---|---|---|---|---|
| Urine | pABG | 267 > 120 | 10 umol/L | CV = 6.06% ($n$ = 10) | 96.90 |
| | | | 100 umol/L | CV = 12.66% ($n$ = 10) | 100.35 |
| | apABG | 309 > 180 | 10 umol/L | CV = 2.89% ($n$ = 10) | 91.84 |
| | | | 100 umol/L | CV = 4.03% ($n$ = 10) | 95.48 |
| | | 309 > 162 | 10 umol/L | CV = 3.59% ($n$ = 10) | 102.40 |
| | | | 100 umol/L | CV = 3.07% ($n$ = 10) | 102.35 |
| Serum | pABG | 267 > 120 | 10 nmol/L | CV = 4.80% ($n$ = 10) | 81.90 |
| | | | 100 nmol/L | CV = 21.72% ($n$ = 10) | 80.40 |
| | apABG | 309 > 180 | 10 nmol/L | CV = 2.54% ($n$ = 10) | 122.80 |
| | | | 100 nmol/L | CV = 20.04% ($n$ = 10) | 96.84 |
| | | 309/162 | 10 nmol/L | CV = 2.02% ($n$ = 10 | 88.94 |
| | | | 100 nmol/L | CV = 12.91% ($n$ = 10) | 92.82 |

catabolites were $11.4 \pm 8.7$ nmol/L, with apABG making 17% and pABG 83% of the total catabolites.

## 4. Discussion

Evidence that folate catabolism proceeds by splitting of folate molecule at C9-N10 bond was initially provided by Futterman and Silverman [10] who demonstrated the enzymatic reduction of folate to tetrahydrofolates followed by spontaneous cleavage to pABG and a pteridine moiety. Murphy and Scott [11] analysed $^{3}$H-labeled compounds excreted in rat urine each day over a 10-day period following an intramuscular administration of [3′,5′,7,9-$^{3}$H]pterylglutamate. They found a number of pteridines and pABG, with the major catabolite consisting of apABG. To demonstrate the suitability of apABG as an indicator of folate catabolism, Geoghegan et al. [12] showed that reverse-phase HPLC of radiolabeled urinary products of rats given [$^{3}$H] and [$^{14}$C] folic acid contained a variety of intact folate as well as products of C9-N10 scission of the folate molecule, pteridines, pABG and apABG. Of all the folate metabolites present in the

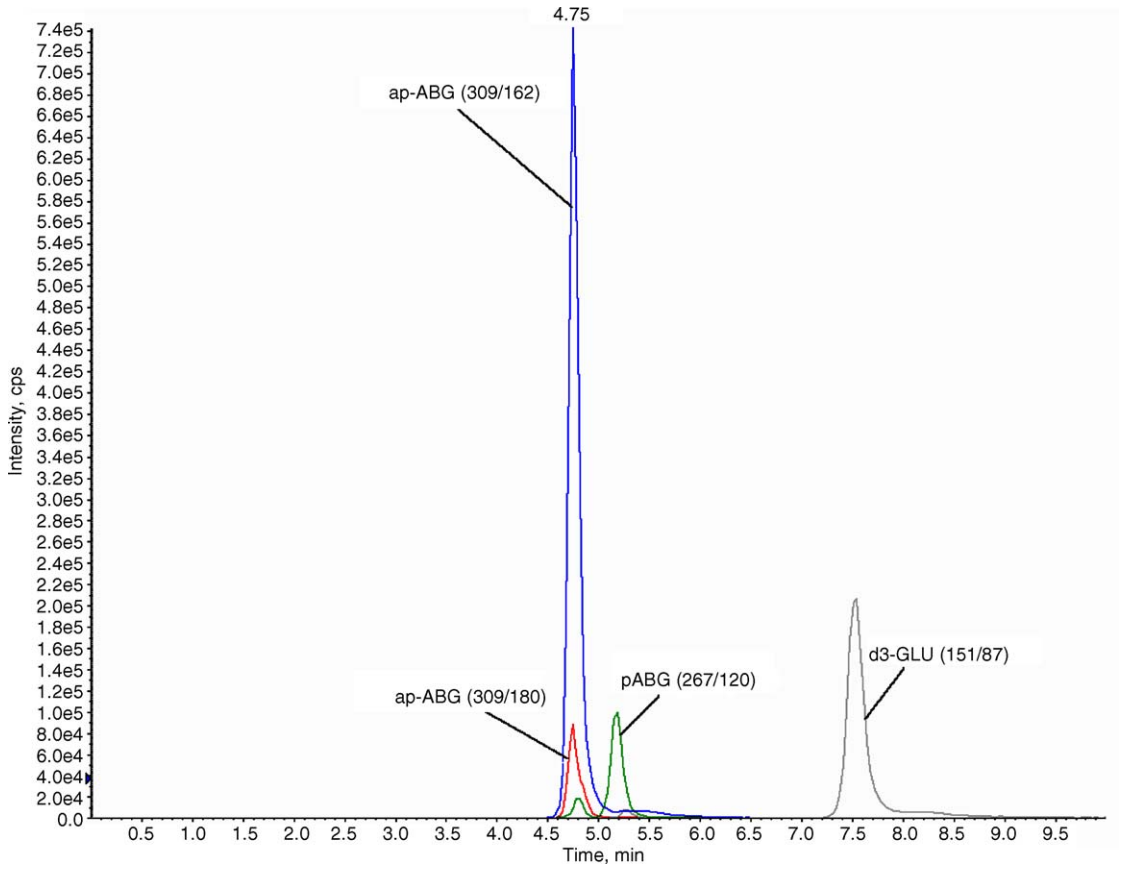


Fig. 4. LC–MS/MS–SRM of pABG, apABG and d3-Glu in 100 umol/L spiked urine.

urine, only apABG persisted at high levels for the 10-day period of the experiment. This finding is corroborated by the works of Connor et al. [13] and Pheasant et al. [14,15].

Serum folate levels in the lab volunteers (35.3 ± 5.8 nmol/L), PTH (27.0 ± 10.4 nmol/L) and prenatal (33.9 ± 22.7 nmol/L) groups were not significantly different from each other ($p < 0.05$) (Fig. 5). These levels are in line with levels found by the National Health and Nutritional Examination Survey (NHANES), 1999–2000 [16]. This study found that median serum folate concentrations for women increased from 10.9 nmol/L (4.8 ng/mL) to 29.5 nmol/L (13.0 ng/mL) from 1988–1994 to 1999–2000.

Serum catabolite levels (Fig. 6) in the lab volunteers, PTH and prenatal groups were also not significantly different from each other ($p < 0.05$). Serum samples collected for prenatal analysis are collected and shipped to the lab at 4 °C. It takes up to 24-hrs for the sample to reach the lab. Once in the lab, the samples were analysed for pABG and apABG after the designated tests (prenatal panel) were done. Thus, analysis of serum catabolites was done approximately 48 h after collection and storage at 4 °C. We believe the degradation of pABG and possible dilution

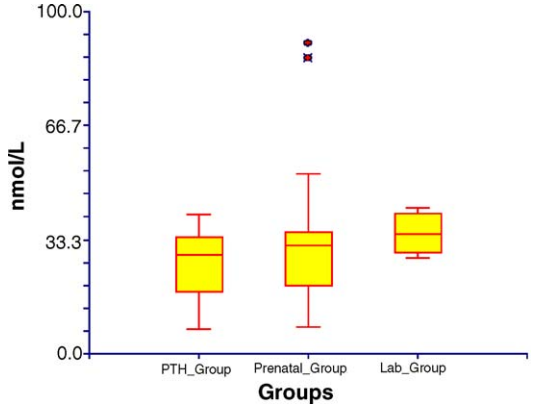


Fig. 5. Box plot of serum folate levels (mean ± S.D. nmol/L) in the respective groups. Solid dots represent outliers, serum folate levels are: PTH_Group ($n = 11$) = 27.0 ± 10.4 nmol/L; Prenatal_Group ($n = 18$) = 33.9 ± 22.7 nmol/L; Lab_Group ($n = 5$) = 35.3 ± 5.8 nmol/L.

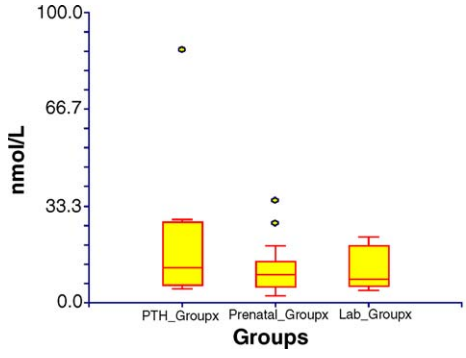


Fig. 6. Box plot of total serum folate catabolites (pABG + apABG) levels (mean ± S.D.) in the respective groups. Solid dots represent outliers. Total serum catabolites levels were: PTH_Group ($n = 11$) = 20.4 ± 23.8 nmol/L; Prenatal_Group ($n = 18$) = 11.4 ± 8.7 nmol/L; Lab_Group ($n = 5$) = 11.9 ± 7.6 nmol/L.

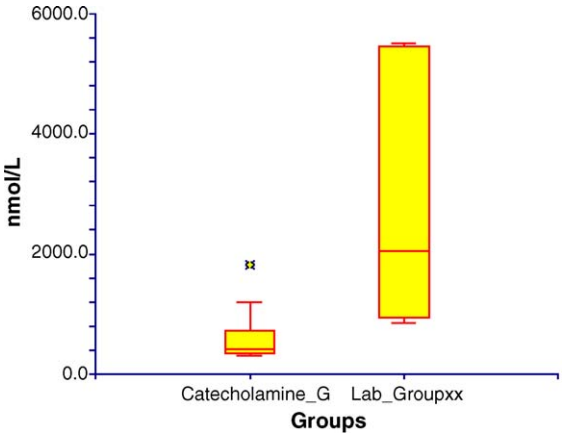


Fig. 7. Box plot of total urinary folate catabolites (pABG + apABG) levels (mean ± S.D. nmol/L) in the respective groups. Solid dots represent outliers. Total urinary folate catabolites levels are: Catecholarnine group ($n = 19$) = 581.8 ± 368.4 nmol/L; Lab group ($n = 5$) = 2.9 ± 2.3 pmol/L.

effect of the increased blood volume during pregnancy could have contributed to the lower levels of serum catabolites seen in this group despite increased folate utilization anticipated in pregnancy [17,18].

Urinary folate catabolite levels (Fig. 7) in the lab volunteers was not significantly different from the catecholamine group ($p < 0.05$) (mean of 581.8 ± 368.4 nmol/L in catecholamine group versus 2.9 ± 2.3 μmol/L in the lab volunteers). This is probably due to the greater standard deviation in the catecholamine group and the fact that catecholamines urine samples are 24-h urine collection. While urine samples collected from lab volunteers were frozen immediately upon collection, the catecholamine samples were stored at 4 °C for 24-h before being frozen. We believe that this storage condition would have led to loss of some catabolites. The levels seen in the lab volunteers, however, are similar to that seen in vivo kinetic studies of folate metabolism [19] and others [15,20]. There was no urine sample available for the other groups.

## 5. Conclusion

We have developed a novel, rapid test for pABG and apABG that can be used in routine clinical assessment of folate catabolism. In this method, both pABG and apABG are assayed simultaneously within the same sample. The samples are acidified with a small amount of 6N HCL (4 μL HCL to 200 μL sample) and precipitated with acetonitrile. Deuterated glutamic acid is added to the samples before HCL acidification and acetonitrile precipitation. It is used as an internal standard because of its similarity to the folate catabolite molecules. Separation of the two compounds is achieved by HPLC and, detection by Multiple SRM of specific ions in MS/MS. Sensitivity of this method is very high with detection of femtomolar (fmol) amounts.

To date, all the methods available for the analysis of folate catabolism are based on extensive sample clean up and use of HPLC. Not only are these methods labour intensive and time consuming, rendering them impractical for use in routine clinical analysis, but they also have limited sensitivity. The objective of this paper was to develop a routine method for quantitative measurement of the folate catabolites *p*-aminobenzoylglutamate and acetamido-*para*-aminobenzoylglutamate in serum and urine using liquid chromatography-tandem mass spectrometry. We are in the process of carrying out further clinical investigation of folate utilization using this novel method. Our method provides ease of analysis with much higher sensitivity, specificity, and versatility regardless of the type of biological sample used, and faster turnaround times.

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