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Reversed-phase high-performance liquid chromatographic method for the quantitation of endogenous folate catabolites in rat urine

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ABSTRACT

We describe a reversed-phase high-performance liquid chromatographic procedure for the analysis of rat urine for *p*-aminobenzoylglutamate (pABGlu) and its acetamido derivative (*p*-acetamidobenzoylglutamate, apABGlu). These two catabolites arise following the *in vivo* cleavage of the folate molecule at the C-9–N-10 bond. Known quantities of high-specific-activity tritiated forms of the catabolites are added as internal standards to aliquots of rat urine. Following preliminary sample clean-up on C_{18} Sep-Pak cartridges, including derivatisation in the case of pABGlu, the urinary extracts are quantitated by HPLC. The present assay makes possible for the first time the determination of endogenous folate breakdown in the rat.

INTRODUCTION

Since the total amount of biologically active folates excreted by man and animals is considerably less than that which is ingested under normal dietary conditions, it has been appreciated for some time that a considerable level of catabolism of folates to inactive breakdown derivatives occurs in mammalian species. Furthermore, it has been suggested that accelerated catabolism of folates may explain folate deficiencies which arise in association with certain clinical conditions or physical states in man including malignancy [1], Crohn's disease [2], anticonvulsant therapy [3,4], alcohol abuse [5,6] and pregnancy [7].

To date, studies of folate catabolism in animals and man have involved the administration of radiolabelled tracers, either [^{14}C]pteroylmonoglutamate ([^{14}C]PteGlu) or [^{3}H]PteGlu, with subsequent analysis of excreted catabolites. Early studies by this laboratory [8] demonstrated that cellular folate catabolism in the rat proceeds by cleavage of the C-9–N-10 bond of the molecule to produce pteridines on one hand, and *p*-aminobenzoylglutamate (pABGlu), along with its acetamido derivative *p*-acetamidobenzoylglutamate (apABGlu), on the other. A similar finding was subsequently reported in man by Krumdieck *et al.* [9] who found radiolabelled pterin and isoxanthopterin in the urine of human volunteers several weeks after administration of [^{14}C]PteGlu, an

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observation consistent with this mechanism of catabolism. While studies such as these involving the administration of radiolabelled folic acid have provided valuable information on identifying the nature and comparative rates of excretion of folate catabolites under defined conditions, they do not allow their absolute levels to be determined.

This paper describes a new HPLC method for the determination of the urinary catabolites pABGlu and apABGlu derived from the endogenous folate pool in the rat, independent of *in vivo* radiolabel administration. The assay involves solid-phase extraction methods followed by HPLC quantitation of excreted catabolites. High-specific-activity tritiated forms of both catabolites are included as internal standards. The synthesis and purification of these radiolabelled standards are described in detail.

EXPERIMENTAL

Materials

[3′,5′,7,9-^{3}H]PteGlu (TRK 212, specific activity 46 Ci/mmol) was purchased from Amersham International (Slough, UK) and used to synthesise [^{3}H]pABGlu and [^{3}H]apABGlu, respectively, as described below. These high-specific radiolabelled compounds were used as internal standards to monitor the recovery of catabolites in analytical samples.

PteGlu and pABGlu were obtained from Sigma (Poole, UK). The method of Baker *et al.* [10] was used to prepare apABGlu from pABGlu, the purity of which was checked by HPLC as described below. Tetrabutylammonium phosphate (PIC reagent A) was prepared in this laboratory from tetrabutylammonium hydroxide (40%, w/w, aqueous), purchased from Sigma. To 33.3 ml of the hydroxide (1.5 *M* as purchased), orthophosphoric acid (BDH, Poole, UK) was added to pH 7.0 and the final volume was adjusted with distilled water to 100 ml to provide a 0.5 *M* stock solution of PIC reagent A. Citrate–phosphate reagent (50 m*M*) was prepared from 50 m*M* disodium hydrogen orthophosphate (BDH) by the addition of 1 *M* citric acid (Sigma) to pH 4.0. Both reagents were stored as stock solutions in dark containers at 4°C for periods of up to one month. All other chemicals were of Analar grade and obtained from Sigma except where otherwise stated. Water was deionized and doubly distilled from glass apparatus. All solvents and solutions for HPLC analysis were filtered through a Millipore filter, pore size 0.45 μm, and vacuum-degassed by sonication before use. Reversed-phase, disposable C_{18} Sep-Pak cartridges were purchased from Waters Assoc.

Apparatus

The HPLC equipment (Waters Chromatography Division, Milford, MA, USA) consisted of a solvent delivery system (Waters Model 510), an injector (Waters Model U6K) and a UV detector (Waters Model 455 LC spectrophotometer) set at 280 nm and at a sensitivity of 0.08 a.u.f.s. Samples were chromatographed using a microparticulate radially compressed reversed-phase column (Waters Z-Module radial compression system equipped with a 10 cm × 0.8 cm I.D Radial-PAK C_{18} cartridge, 10 μm packing). A guard column module containing a disposable guard column insert (RCSS Guard-PAK) was used to protect the analytical column from damage due to irreversibly adsorbed particulates and to improve its lifetime. The detector signal was monitored on a strip chart recorder (Omniscribe D5000, Houston Instruments, Austin, TX, USA) with an output of 10 mV.

Radioactivity measurements were determined using a Tri-Carb 1500 liquid scintillation analyser (Packard Canberra, Reading, UK). This system has inbuilt quench correction standards which automatically compensate for sample quenching. Samples for counting were dissolved in Hydroflour Universal liquid scintillation cocktail (National Diagnostics, Manville, NJ, USA).

Chromatographic conditions

Chromatography was carried out using an isocratic elution at 2 ml/min at room temperature with one of two buffer systems, having activated the HPLC column with methanol (20 min) followed by distilled water (20 min). For pABGlu

analyses, a citrate–phosphate (0.1 *M*, pH 4.0)–acetic acid (1%)–methanol (70:21:9) buffer was used. For apABGlu analyses, the mobile phase consisted of 25% methanol–tetrabutylammonium phosphate (PIC reagent A, 5 m*M*)–ammonium dihydrogen orthophosphate (0.01 *M*), pH 7.0. Buffers were freshly prepared on a daily basis for HPLC analysis. Following a 20-min equilibration period to establish a suitable baseline, samples (100 μl) were injected using a glass syringe (Microliter, Hamilton).

Synthesis and purification of radiolabelled standards

[^{3}H]pABGlu. [^{3}H]pABGlu was prepared from [3′,5′,7,9-^{3}H]PteGlu by reductive cleavage as follows. To 125 μl of [^{3}H]PteGlu (100 μCi) were added 50 μl of HCl (5 *M*) and 50 μl of a zinc dust (BDH) suspension (1 g of zinc per 2 ml of 0.5% gelatin) into a 1.5-ml microcentrifuge polypropylene tube. The mixture was vortex-mixed (3 min), allowed to stand (15 min, room temperature), then centrifuged to remove excess zinc. The radioactive supernatant was carefully removed and applied to HPLC, collecting eluates in 1-ml fractions using a Frac-100 fraction collector (Pharmacia, Uppsala, Sweden). An early radiolabelled peak corresponding to pteridine material was discarded; the second peak, [^{3}H]pABGlu, eluted at 8.0 min. Fractions containing the latter peak were pooled and stored at −20°C in 100-μl aliquots until required for use.

[^{3}H]apABGlu. [^{3}H]apABGlu was prepared by taking a portion of the newly synthesized [^{3}H]pABGlu above and performing an acetylation reaction as described by Baker *et al.* [10] with the following modifications. The [^{3}H]pABGlu (25 μCi, 5 ml) was first concentrated by solid-phase adsorption, a step which was facilitated by converting it to the diazotised derivative by coupling with naphthylethylenediamine, *i.e.* the Bratton–Marshall reaction [11]. This involved acidification of 5.0 ml of the radioactive material by the addition of 100 μl of HCl (5 *M*), then the sequential addition of 100 μl of each of the following Bratton–Marshall reagents, allowing 5 min between each reagent and the addition of the next: sodium nitrite (1%); ammonium sulfamate (5%); N-1-naphthylethylenediamine (1%). The last of these was freshly prepared immediately before use, while the first two reagents once prepared were stored in dark containers for periods of up to six months. (The usual purple colour of the diazotised derivative was not observed due to the high specific activity of the [^{3}H]pABGlu used.) After 30 min incubation, the diazotised radiolabelled derivative was applied to a pre-activated (3 ml of methanol followed by 5 ml of water) C_{18} Sep-Pak cartridge. Having washed the bound material with 10 ml of distilled water, it was eluted with 3 ml of methanol using a disposable syringe. [^{3}H]pABGlu was regenerated by reductive cleavage (50 μl of 5 *M* HCl followed by 50 μl of zinc dust suspension). The mixture was vortex-mixed for 3 min, then centrifuged, leaving a clear supernatant containing methanol–suspended [^{3}H]pABGlu which was carefully transferred to a conical glass tube and evaporated to dryness under nitrogen at 37°C. Once evaporated to dryness, the radiolabelled extract was converted to its acetamido derivative by incubating at 22°C for 24 h with 34 μl of acetic acid (50%, v/v) and 5 μl of acetic anhydride. The volume of the reaction mixture was adjusted to 100 μl by the addition of distilled water, then applied to HPLC to isolate the purified [^{3}H]apABGlu which eluted at 16 min. An insignificant peak representing unreacted [^{3}H]pABGlu was detectable but 91% of the radioactivity was recovered as [^{3}H]apABGlu.

Urine collection

Urine samples (24 h) were obtained from male Wistar rats weighing 252–268 g, housed individually in wire-bottomed Techniplast metabolic cages under controlled environmental conditions of temperature (20°C), relative humidity (50%) and photoperiod (12 h light–dark schedule). Urine samples (approximately 10 ml per rat per day) were collected in ascorbic acid (1% with respect to the urine) to protect any labile reduced folates from oxidative changes [12]. On terminating each collection, the urinary volume was adjusted to 50 ml, rinsing with distilled water contained in a wash bottle to ensure that any dried urine depos-

ited on the apparatus was included in that collection. Once collected, urine samples were stored in 50-ml volumes at −20°C until required. Animals were fed the AIN-76 purified diet [13], providing a folic acid content of 1 mg/kg (approximately 25 μg per rat per day) except where otherwise stated.

Measurement of urinary folate catabolites

Pre-HPLC procedure for pABGlu. A known quantity of [^{3}H]pABGlu (50 000 dpm) was added to duplicate 10-ml aliquots of rat urine as an internal radiolabelled standard by which percentage recovery was monitored. The pH of each sample was adjusted to 7.0 with NaOH (1 *M*) before applying it onto a C_{18} Sep-Pak cartridge which had been pre-activated by pushing through the following with a disposable syringe: 3 ml of methanol followed by 5 ml of distilled water followed by 2.5 ml of PIC reagent A (5 m*M*). The sample was allowed to filter through the cartridge by gravity, and the filtrate (10 ml) was collected in a 25-ml plastic Sterilin container. The cartridge was then washed with 10 ml of distilled water, the filtrate was again collected and combined with the initial filtrate, discarding the used cartridge and its bound material. The filtrate (20 ml) was acidified by the addition of 800 μl of HCl (5 *M*) and the Bratton Marshall [11] reaction was performed as described above, but with the minor modification that the volume of each of the three reagents used was 200 μl. The development of a purple colour following the addition of the final reagent signified the conversion of pABGlu to its diazotised derivative. After 30 min incubation, this material was loaded onto a second C_{18} Sep-Pak cartridge which had been pre-activated by pushing through 3 ml of methanol followed by 5 ml of distilled water with a disposable syringe, and allowed to filter through by gravity. The filtrate was discarded. The cartridge was washed with 10 ml of distilled water and the filtrate was again discarded. The diazotised material retained on the cartridge was eluted with 1.5 ml of acidified methanol [one drop of HCl (5 *M*) to 10 ml of methanol] using a disposable syringe. The eluate was evaporated to dryness under nitrogen at 37°C (approximately 45 min) in a plastic Sterilin container, then reconstituted in 400 μl of HCl (0.3 *M*). Regeneration of pABGlu was accomplished by the addition of 50 μl of zinc dust suspension, followed by 3 min vortex-mixing. The reaction mixture was centrifuged for 3 min and a clear supernatant was removed for HPLC analysis.

Pre-HPLC procedure for apABGlu. To duplicate 10-ml aliquots of urine was added [^{3}H]apABGlu (50 000 dpm) as a means of monitoring percentage recovery during this procedure. The pH of each sample was adjusted to pH 4.0 using citric acid (1 *M*) before loading onto a pre-activated (3 ml of methanol followed by 5 ml of distilled water followed by 5 ml of citrate–phosphate reagent, each pushed through using a disposable syringe) C_{18} Sep-Pak cartridge. The sample was allowed to filter through the cartridge by gravity and the filtrate was discarded. The cartridge was then rinsed with 3 ml of citrate–phosphate reagent, 50 m*M* (pushed through using a disposable syringe) and the filtrate was again discarded. The material retained on the cartridge was eluted with 1.5 ml of methanol using a disposable syringe, then evaporated to dryness under nitrogen at 37°C in a plastic Sterilin container. The extract was reconstituted in 10 ml of PIC reagent A, was loaded onto a second pre-activated (3 ml of methanol followed by 5 ml of distilled water followed by 2.5 ml of PIC reagent A) C_{18} Sep-Pak cartridge and allowed to filter through by gravity. The filtrate was discarded, the cartridge was rinsed with 3 ml of PIC reagent A and the second filtrate was discarded. The material retained on the cartridge was eluted using a disposable syringe in three consecutive elutions with PIC reagent A buffer. The eluate arising from the first elution [1 ml of 25% methanol–PIC reagent A (5 m*M*)–ammonium dihydrogen orthophosphate (0.01 *M*), pH 7.0] was discarded. The second elution [0.5 ml of 30% methanol–PIC reagent A (5 m*M*)–ammonium dihydrogen orthophosphate (0.01 *M*), pH 7.0] was again discarded. The third elution [0.5 ml of 30% methanol–PIC reagent A (5 m*M*)–ammonium dihydrogen orthophosphate (0.01 *M*), pH 7.0] was retained for HPLC analysis.

Quantitation by HPLC. Starting from initial 10-ml urinary volumes, the pre-HPLC sample preparation procedures resulted in 400- and 500-μl extracts for pABGlu and apABGlu, respectively. In each case, a 100-μl extract was analysed by HPLC under the conditions described earlier. For both procedures the catabolite in the injected sample was quantitated by relating the peak height (mm) to a standard plot (10–100 ng). The computed value for each urinary extract was adjusted to correct for percentage recovery and for total urinary volume to give a final value of excreted catabolite (μg per rat per day). The percentage recovery of each analytical sample was monitored by the appropriate internal radiolabelled standard, [^{3}H]pABGlu or [^{3}H]apABGlu, a known quantity of which (50 000 dpm) was added at the start of each procedure. In both procedures, a percentage recovery was calculated for each analytical sample by collecting the radioactivity of the HPLC extract post-column and relating this to the total radioactivity added (50 000 dpm) at the start of the procedure. Overall recoveries were 47 and 42% for pABGlu and apABGlu procedures, respectively.

RESULTS

HPLC performance

Fig. 1A is a typical chromatogram of an extract of rat urine for pABGlu analysis. The validity of the analytical pABGlu peak was confirmed by various means. Firstly, by spiking the HPLC extract with a known quantity of pABGlu standard before re-injecting, an increase in peak height corresponding to the concentration of standard added was observed (Fig. 1B). To rule out the possibility of the presence of another compound co-chromatographing with pABGlu in analytical samples, dual monitoring of the analytical peak at alternative wavelengths was observed. Following the monitoring of a series of pABGlu standards at wavelengths 280 and 254 nm, a peak-height ratio of 3.48:1 (280/254 nm) was established. Measurement of the analytical pABGlu peak at these wavelengths revealed an identical peak-height ratio.

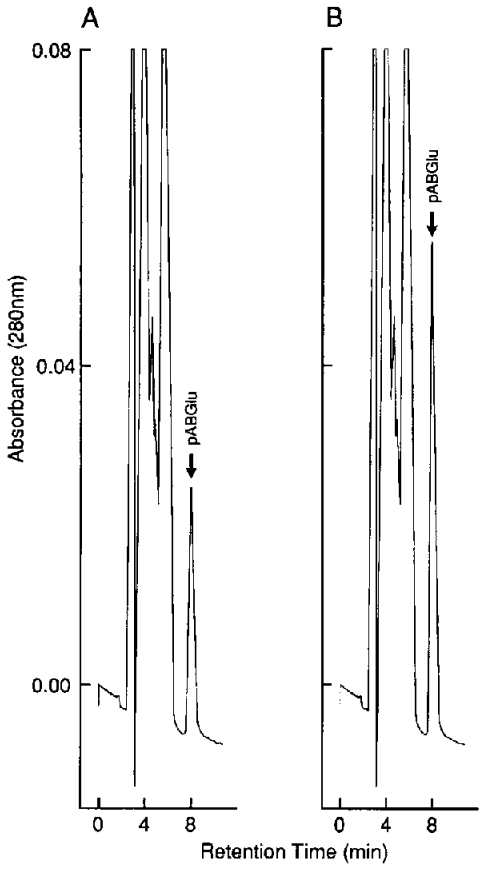

Fig. 1. Representative chromatograms of rat urine extracts separated by reversed-phase HPLC showing (A) endogenous pABGlu peak and (B) elevation of the peak following re-injection of the extract spiked with a known quantity of pABGlu standard. For sample preparation and HPLC conditions see text.

Fig. 2A is a typical HPLC profile of a rat urine extract for apABGlu determination. The authenticity of the analytical peak was established as follows. Firstly, a repeat injection of the HPLC extract, spiked with a known quantity of apABGlu standard, resulted in a corresponding elevation in the analytical apABGlu peak height (Fig. 2B). Further validation was established by the observation that the apABGlu peak could be eliminated by performing a deacetylation procedure (acid hydrolysis, by boiling for 1.5 h with 0.4 *M* HCl) on the HPLC extract before re-injection (Fig. 2C). When the radioactivity in 1-ml fractions of eluate was determined, there was a corre-

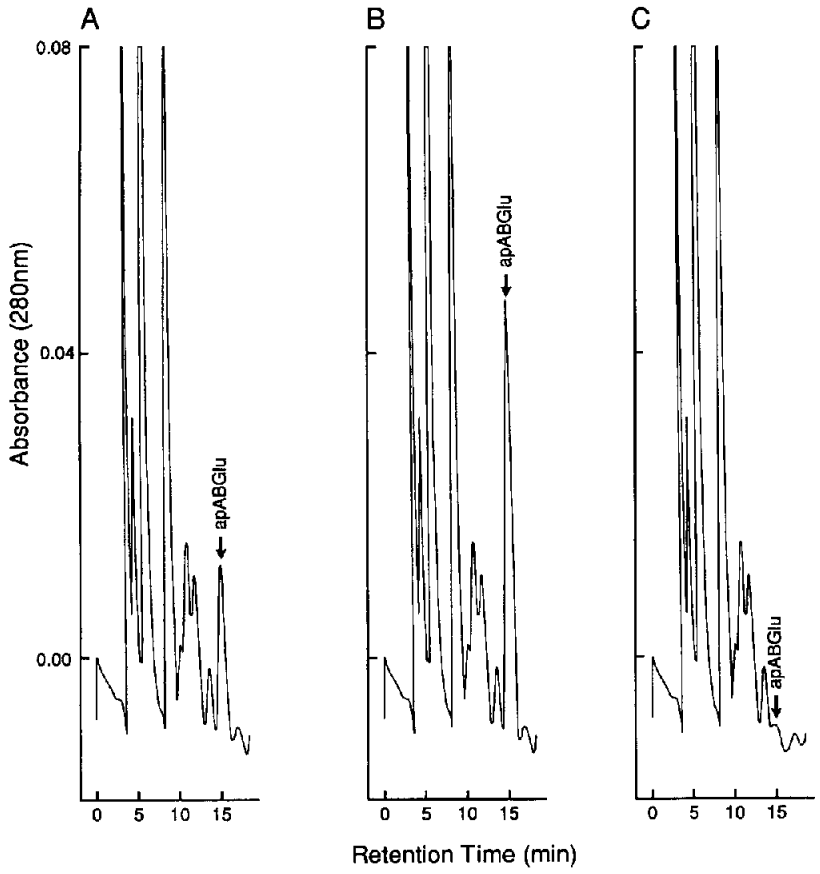

Fig. 2. HPLC chromatograms of rat urine extracts showing (A) endogenous apABGlu peak, (B) elevation of the peak following spiking of the extract with a known quantity of apABGlu standard and (C) disappearance of the apABGlu peak following a deacetylation procedure by acid hydrolysis (boiling for 1.5 h with 0.4 *M* HCl) to degrade it. Chromatographic conditions were as described in the text.

sponding disappearance of the [^{3}H]apABGlu peak which could be accounted for in a [^{3}H]pABGlu peak, the expected deacetylation product.

Assay performance

A representative experiment to show intra-assay precision gave values for six determinations of pooled rat (n = 4) urine (expressed as mean ± S.D.) of 0.38 ± 0.02 and 1.40 ± 0.03 μg per 10 ml for pABGlu and apABGlu analyses, respectively. Inter-assay precision values, assessed by analysing the same pool on six occasions over a two-week period, were 0.42 ± 0.05 and 1.32 ± 0.08 μg per 10 ml for pABGlu and apABGlu, respectively.

Recovery experiments were carried out on pooled rat (n = 4) urine, and representative results are given in Table I. Aliquots (10 ml) were spiked with a known quantity of standard and taken through each assay procedure. The accuracy of the techniques is demonstrated by the ability of each assay to satisfactorily recover the catabolite being measured and be unaffected by added quantities of the other catabolite.

The effect of dietary folic acid level was determined (Table II). Where a diet free of added folic acid was fed, apABGlu excretion remained unchanged, while pABGlu excretion fell to levels below the limit of detection of the assay (*i.e.* < 1.0 μg per rat day).

TABLE I

REPRESENTATIVE RECOVERY OF ADDED STANDARDS TO 10-ml ALIQUOTS OF POOLED RAT URINE

	Recovery[a] (μg per 10 ml)	
	pABGlu	apABGlu
pABGlu assay		
No added standard	0.41	–
Plus 1 μg added pABGlu	1.37	–
Plus 1 μg apABGlu	0.34	–
apABGlu assay		
No added standard	–	1.39
Plus 1 μg apABGlu	–	2.30
Plus 1 μg pABGlu	–	1.42

[a] Based on duplicate analysis.

TABLE II

REPRESENTATIVE URINARY EXCRETION OF FOLATE CATABOLITES BY INDIVIDUAL RATS WITH AND WITHOUT FEEDING FOLIC ACID

Catabolite	Urinary excretion[c] (μg per rat per day)			
	1	2	3	4
Folic acid, 1 mg/kg diet[a]				
pABGlu	1.8	1.5	1.9	1.7
apABGlu	6.1	6.0	5.7	5.8
	5	6	7	8
Folic acid-free diet[b]				
pABGlu	<1.0	<1.0	<1.0	<1.0
apABGlu	5.9	6.1	5.9	5.8

[a] The AIN-76 purified diet [13] provided 1 mg/kg added folic acid (*i.e.* approximately 25 μg per rat per day).

[b] Folic acid-free diet is taken to mean the above regime with the omission of folic acid from the vitamin mixture. The "folic acid free" regime has been found to contain residual levels of 0.14 [14] and 0.13 [15] mg folate per kg.

[c] Each analysis was performed in duplicate on eight 24-h samples collected following an equilibration period of one week of feeding the experimental diet. Serum folates were measured by microbiological assay [16] to check that the appropriate diet had been fed. Expressed as mean ± S.D. these were 17.9 ± 2.2 and 10.5 ± 1.9 μg/l for folic acid fed and folic acid free dietary groups, respectively.

DISCUSSION

The methodology described in the present report makes possible for the first time the determination of endogenous folate catabolites in rat urine. This group has previously demonstrated [8] that after a sufficient equilibration period the principle catabolite of a tracer dose of [^{3}H]PteGlu in the rat was apABGlu, arising through *in vivo* acetylation of an initial catabolite pABGlu, a small amount of which was also detected in the urine. It was thus concluded that the principle mechanism of cellular folate catabolism proceeded by cleavage of the C-9–N-10 bond, and not by excretion of inactive forms of the vitamin which still contained the intact folate skeleton as had been postulated earlier by others [17,18]. More recent investigations of this nature by our group [19] have provided additional evidence which support this mechanism of folate catabolism. It was shown that when [^{3}H]PteGlu was administered orally to rats, an acetamido derivative of *p*-aminobenzoic acid (apABA) was identified in addition to pABGlu and apABGlu. When the dose was administered via the parenteral route, no apABA could be detected. This suggests that the apABA reported by earlier workers [17] was of gut microfloral, and not of mammalian origin. Therefore, there is good experimental evidence to rely on the measurements of pABGlu and apABGlu as indicators of mammalian folate catabolism. While previous studies have enabled these compounds to be identified in rat urine, the present methodology allows their quantitation.

The procedures described for the determination of urinary folate catabolites in the rat are based on solid-phase extraction techniques followed by HPLC quantitation. In the case of pABGlu analysis the pre-HPLC sample clean-up is enhanced by including a diazotisation step which, by altering its chemical characteristics, facilitates the retention of pABGlu on a C_{18} Sep-Pak cartridge. Crucial to both methods is the inclusion of high-specific-activity radiolabelled forms of these catabolites, known quantities of which are added as internal standards to individual aliquots of rat urine at the start of each proce-

dure. In each case, the radioactivity in the analytical HPLC peak is related to the total amount added in order to establish a percentage recovery for each analytical sample. Computed values for individual extracts are then adjusted to correct for recovery. It is important to note that the use of such high-specific-activity radiolabelled standards means that they do not contribute to the UV peaks seen with analytical extracts, their concentration being insignificant compared to that of the corresponding endogenous urinary catabolite.

The techniques used have been validated for HPLC performance (Figs. 1 and 2) and overall assay performance, showing good recoverability for both pABGlu and apABGlu (Table I). The 24-h data on individual rats show that an inescapable level of folate catabolism exists, in that apABGlu was excreted in measurable levels in all animals, irrespective of dietary folate intake (Table II). The finding that there was no significant difference in apABGlu excretion in rats fed a diet providing 1 mg/kg added folic acid compared to those fed a diet which contained no added folic acid, is consistent with this catabolite being derived from the endogenous folate pool. However, the level of urinary pABGlu seemed dependent on dietary and, in turn, serum folate level (Table II, footnote), its excretion falling to levels below the limit of detection of the assay (*i.e.* <1.0 μg per 24 h) when rats were fed a diet free of added folic acid. A possible explanation for this finding might be that unacetylated pABGlu is present in urine as an artifact, whereas urinary apABGlu is a true reflection of cellular catabolism of folate. When the mechanism of folate catabolism was first elucidated by this group [8] and in subsequent reports identifying the products of folate catabolism in the rat [20], apABGlu was shown to be the principle catabolite, arising through *in vivo* acetylation of pABGlu occurring in the liver, kidney or both. The present findings are consistent with this mechanism and suggest that measurement of urinary apABGlu alone may be valid as a measure of mammalian catabolism of folate.

The development of this methodology has important clinical and nutritional applications. In future studies, the rat may be used as a model to examine the rate of folate catabolism under various clinical conditions and physiological states which are associated with human folate deficiency, but for which the mechanism remains unclear. It is possible for instance, that certain of these conditions may be the result of an elevation in the normal rate of folate catabolism. This explanation has been previously proposed for various folate-deficient states [1–7], but until now the methodology to address them in a satisfactory manner has not existed.

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