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ORIGINAL ARTICLE

An isotope-dilution, GC–MS assay for formate and its application to human and animal metabolism

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Abstract Formate, a crucial component of one-carbon metabolism, is increasingly recognized as an important intermediate in production and transport of one-carbon units. Unlike tetrahydrofolate-linked intermediates, it is not restricted to the intracellular milieu so that circulating levels of formate can provide insight into cellular events. We report a novel isotope-dilution, GC–MS assay employing derivatization by 2,3,4,5,6-pentafluorobenzyl bromide for the determination of formate in biological samples. This assay is robust and sensitive; it may be applied to the measurement of formate in serum, plasma and urine. We demonstrate how this method may be applied by providing the first characterization of formate levels in a human population; formate levels were higher in males than in females. We also show how this procedure may be applied for the measurement of in vivo kinetics of endogenous formate production in experimental animals.

Keywords Serine · Glycine · One-carbon metabolism · Folate · Methanol

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Introduction

Formate, which plays a major role in one-carbon metabolism, is found in the plasma at relatively low concentrations (Lamarre et al. 2012). It is produced by a number of processes, particularly involving amino acid metabolism in both the cytoplasm and mitochondria. In the cytoplasm formate is produced by tryptophan catabolism, cytochrome P-450 demethylation reactions (including those in the pathway of cholesterol synthesis and the α-oxidation of branched fatty acids) and methylthioadenosine recycling (Lamarre et al. 2013). Formate is also produced during methanol catabolism; indeed, formic acid is considered to be responsible for much of the toxicity of methanol intoxication (Liesivuori and Savolainen 1991; Kapur et al. 2007). Elegant work from Appling's laboratory showed that mitochondria can produce formate. This occurs via the catabolism of serine, glycine, sarcosine and dimethylglycine which results in the production of 5,10-methylene-THF (Barlowe and Appling 1988; Lamarre et al. 2013). This methylene-THF may be oxidized to 10-formyl-THF from which formate may be produced by 10-formyl-THF synthetase. Mitochondrial formate is then released to the cytosol where it is incorporated into 10-formyl-THF and used for purine synthesis; cytosolic 10-formyl-THF may be further reduced to 5,10-methylene-THF and 5-methyl-THF which are used, respectively, for thymidylate synthesis and the remethylation of homocysteine to methionine (Barlowe and Appling 1988). Quantitative aspects of the provision of one-carbon groups from serine and glycine in humans are provided by Davis et al. (2004) and Lamers et al. (2009). Formate catabolism is not fully understood, but it is clear that both folate-dependent and catalase-dependent mechanisms occur (Tibbetts and Appling 2010). Figure 1 provides an outline of formate metabolism.

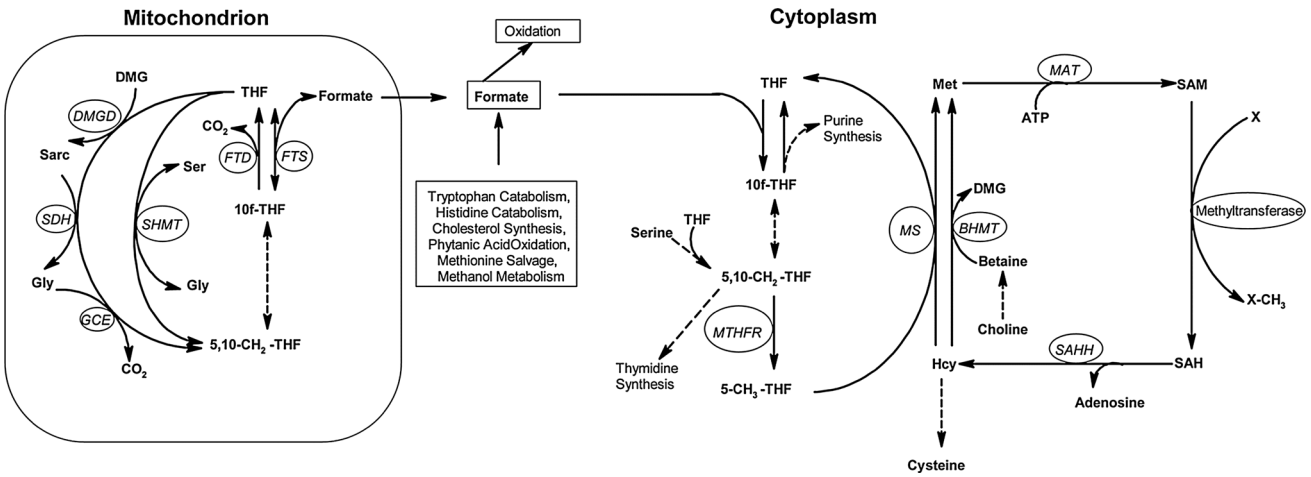

Fig. 1 Outline of formate metabolism

Formate metabolism has become of considerable interest in recent years. Stover's group has shown that mice in which the cytoplasmic serine hydroxymethyltransferase (SHMT) has been knocked out are viable, suggesting a major role for mitochondrial mechanisms in the provision of one-carbon groups (MacFarlane et al. 2008); in addition, nuclear SHMT may provide one-carbon groups for thymidylate synthesis (Anderson and Stover 2009). Recent work by Narisawa et al. (2012) has shown that mutations in genes that code for subunits of the glycine cleavage enzyme (GCS) predispose to neural tube defects in humans and in mice; GCS, a mitochondrial enzyme, produces 5,10-methylene-THF (and ultimately formate) from glycine. Appling's laboratory has reported that knockout of the *mthfd1 l* gene, which codes for the monofunctional mitochondrial 10-formyl-THF-synthetase (the final mitochondrial enzyme in the production of formate) is embryologically lethal (Momb et al. 2013). These embryos display both aberrant neural tube closure and growth delay. Critically, they also found that providing sodium formate in the drinking water of these pregnant mice decreased the incidence of NTDs and partially rescued the growth defect evident in embryos lacking Mthfd1 l activity. Further evidence for a critical role of formate in one-carbon metabolism is provided by our study on vitamin B_{12} and folate deficiency; rats which were deficient in either vitamin had markedly elevated plasma and urinary formate, suggesting that formate levels may be a useful indicator of the nutritional status of these vitamins in humans (Lamarre et al. 2012). A recent metabolomics study of hypertension in a large population (4,630 participants) of different ethnic backgrounds and eating a variety of diets found an association between 24-h urinary formate excretion and blood pressure: hypertension was associated with low urinary formate (Holmes et al. 2008). Finally, formate levels may serve as a useful means of monitoring possible inhibition of methionine synthetase (and induction of a functional deficiency of both folate and vitamin B_{12}) by trichloroethylene, a commonly used industrial de-greasing agent (Green et al. 2004).

Because of this recent interest in formate, we anticipated a need for a new assay that could be applied to human samples. Such a method should be sensitive; it should be suitable for the analysis of the large numbers of samples (many thousands) generated in human epidemiological studies. The method should be equally applicable to serum, plasma and urine and should not be affected by the use of different anti-coagulants. The new method should facilitate studies of whole body formate kinetics involving stable isotopes, which means that it must involve mass spectrometry. We report in this paper a GCMS procedure, which fulfills the criteria outlined above; the method is sensitive to 8 μM and is highly reproducible. We also present novel information on the plasma formate levels of a cohort of 130 human subjects. We also show how the method may be employed, using ^{13}C-formate as a tracer, to study whole body formate kinetics in the rat.

Materials and methods

Materials

All materials were of the highest purity. $Na_2HPO_4{\cdot}7H_2O$ (S373-500) and $NaH_2PO_4{\cdot}H_2O$ (S369-500) were obtained from Thermo Fisher Scientific (catalog numbers are provided in brackets). 2,3,4,5,6-Pentafluorobenzyl bromide (PFBBr) (101052-25G), hexane (34859-1L) and acetone (650501-1L) were purchased from Sigma-Aldrich. Sodium formate (^{13}C, 99 %) (CLM-583-1) and sodium acetate (1,2-$^{13}C_2$, 99 %) (CLM-440-1) were purchased from Cambridge Isotope Laboratories. Solutions for calibration

were prepared from sodium formate (>99 %) obtained from Sigma-Aldrich (71539).

Solutions

The following solutions were prepared:

(i) Solution A. Alkylating reagent. 100 mM PFBBr in acetone.
(ii) Solution B. Sodium ^{13}C-formate. 1 mM sodium ^{13}C-formate in HPLC-grade water.
(iii) Solution C. Stock phosphate buffer. 0.5 M phosphate buffer, pH 8.0.
(iv) Solution D. Working buffer. 10 mL of stock phosphate buffer (Solution C) plus 3 mL of standard solution of sodium ^{13}C-formate (Solution B).

In situations when ^{13}C-acetate was used as the internal standard instead of ^{13}C-formate, Solution E replaced Solution B.

(v) Solution E. Sodium ^{13}C-acetate. 1 mM of sodium $^{13}C_2$-acetate.

Alkylation procedure

The alkylation procedure was carried out in capped, 1.5-mL polypropylene centrifuge tubes [Thermo Fisher Scientific (05-407-10)]. To each vial 20 μL of Solution D (working buffer), 50 μL of sample (serum, plasma, urine or standard) and 130 μL of Solution A (alkylating reagent) were added, vortexed for 1 min and incubated at 60° in a water bath for 15 min. After this, the tubes were allowed to cool at room temperature for a few minutes, 330 μL of *n*-hexane was added, and tubes were vortexed for 1 min after which two layers were allowed to separate. Finally, 200 μL of the organic phase (top layer) was transferred to glass inserts (Thermo Fisher Scientific, #200670) and analyzed by GC–MS.

Instrumentation, GC–MS and quantitation

A Thermo Fisher TRACE GC ULTRA equipped with an ISQ mass selective detector and an AS3000 auto-sampler were used. The GC separation was carried out by injecting a 1-μL aliquot of the extracted and derivatized sample, using an Agilent DB-225 J&W GC column (inner diameter 0.25 mm, 30 m in length, with a coating thickness of 0.25 μm) and maintained under the following GC conditions: carrier gas, He; gas flow 1.5 mL/min; inlet temperature 220 °C; transfer line temperature 300 °C; ion source temperature 300 °C. The GC temperature program was then initiated by holding the temperature of the column at 50 °C for 2 min, followed by an increasing temperature gradient at a rate of 30 °C/min to a final temperature of 220 °C. The temperature was then returned to 50 °C for a new injection. The run time from injection to injection of subsequent samples was 12 min. MS was carried out in the electron impact ionization mode, monitoring *m/z* at 226, 227, and 242 (representing, respectively, the PFBBr derivatives of ^{12}C-formate, ^{13}C-formate, and $^{13}C_2$-acetate).

Results were quantitated by reference to a standard curve containing 0, 25, 50, 75, 100, 250 μM formate, plotted against the ^{12}C/^{13}C-formate ratio on the *y*-axis. The r^2 for the standard curve was invariably greater than 0.99. The measurement of endogenous formate production was carried out on a 180 g, male Sprague–Dawley rat in the fed state. The rat was anesthetized with isoflurane in oxygen (5 % for induction and 2 % for maintenance). The animal was infused via the left iliac vein at a rate of 9.6 μmol of ^{13}C-formate in 0.9 % saline per hour. Blood collection (200 μL per time point) was via a catheter in the right iliac artery. Patency of the arterial catheter was maintained by flushing it with a small volume of heparin–saline (1,000 units heparin per mL saline).

Ethical approval for human and animal experiments

Ethical approval for analysis of the human samples was provided by the Health Research Ethics Authority of Newfoundland and Labrador. The animal study was approved by Memorial University's Institutional Animal Care Committee, in accordance with the guidelines of the Canadian Council on Animal Care.

Expression of data and statistical analysis

Data are presented as mean ± standard deviation. The effects of hemolysis on serum formate levels were analyzed by means of a one-way ANOVA. The difference between the formate levels in men and women was analyzed by Student's *t* test.

Results and discussion

We modified the GC–MS method for formate described by Kage et al. (2004) in several important ways. First of all, we included ^{13}C-formate in the samples so as to permit an isotope-dilution assay. This enabled us to dispense with the internal standard (1,3,5-tribromobenzene) employed by Kage et al. (2004) which elutes considerably later in the gas chromatogram than does the formate derivative. This may account for the fact that we achieved higher sensitivity (8 μM compared with 20 μM) than did Kage et al. (2004). This is a critical improvement as our values for human sera ranged from 17 to 65 μM. Secondly, we shortened the

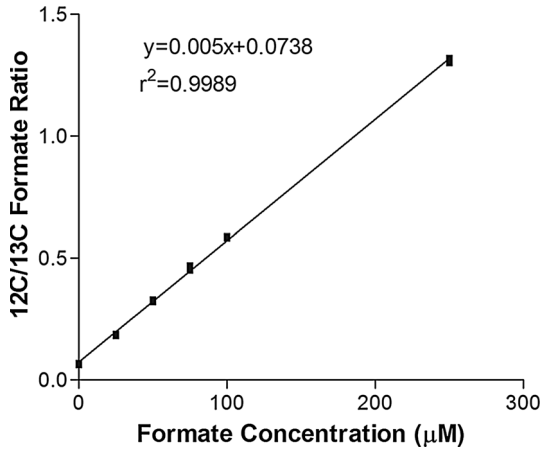

Fig. 2 Standard curve: formate concentration is plotted against the $^{12}C/^{13}C$-formate ratio

derivatization time to 15 min which reduced the magnitude of the blank signal. The value of the blank was the same regardless of whether the standards were made up in water or in saline. The blank was affected by the analytical reagents, some batches giving lower blanks than others. In particular, we obtained smaller blanks with PFBBr from Sigma-Aldrich than from some other suppliers. Thirdly, we carried out the derivatization at a pH of 8.0, as recommended (Tomcik et al. 2011), so as to improve the stability of the formate ester of PFBBr under alkaline conditions. With these modifications, the assay gave robust and reproducible standard curves for formate (Fig. 2), being linear to 500 μM. When four standard curves were run on the same day, the equations describing the lines were (1) $y = 0.0049x + 0.0854$, (2) $y = 0.0050x + 0.0757$, (3) $y = 0.0050x + 0.0814$, and (4) $y = 0.0050x + 0.0738$. The r^2 was invariably greater than 0.99. Figure 3 shows a typical chromatogram of a plasma formate assay, monitoring at *m/z* 226 for ^{12}C-formate and 227 for ^{13}C-formate. We computed an instrument detection limit (IDL) of 8 μM, as calculated from the equation below, where Y_b is the blank value and S_b is the standard error of the regression line (Miller and Miller 2010):

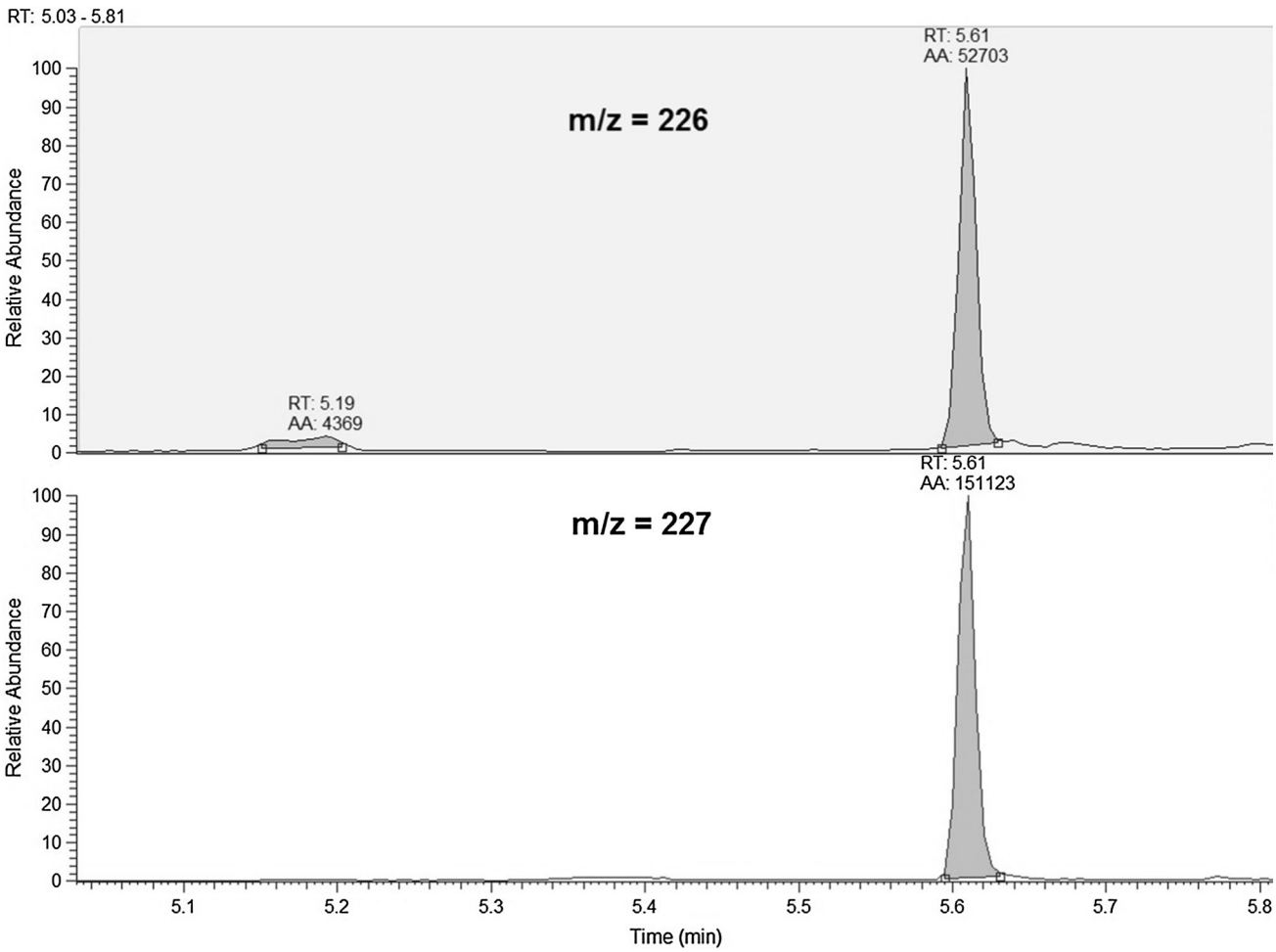

Fig. 3 A typical chromatogram from a plasma formate assay, monitoring at *m/z* 226 for ^{12}C-formate and 227 for ^{13}C-formate

Table 1 Validation of formate assay

(A)	Effect of anticoagulant on formate concentration (μM)		
	Serum	40.0 ± 3.9 (6)	
	EDTA plasma	41.4 ± 5.8 (6)	
	Heparinized plasma	40.5 ± 5.7 (6)	
(B)	Recovery of formate added to serum (μM)		
	Serum	36.7 ± 1.4 (3)	
	Serum + 25 μM formate	63.1 ± 1.5 (3)	102 % recovery
	Serum + 50 μM formate	91.9 ± 1.8 (3)	106 % recovery
(C)	Effect of hemolysis on serum formate concentrations (μM)		
	Percent hemolysis		
	0	37.9 ± 0.6 (5)	
	1	41.4 ± 3.1 (5)	
	2	40.3 ± 0.9 (5)	
	3	42.3 ± 2.6 (5)*	
	4	42.3 ± 2.7 (5)*	
	5	41.0 ± 2.1 (5)*	

Data are presented as mean ± SD (n). There was no significant difference between formate levels in serum and either the EDTA or heparinized plasma. The hemolysis data were analyzed by means of a one-way ANOVA

The *asterisks* significant difference ($P < 0.05$) from the non-hemolyzed samples

$$IDL = Y_b + 3S_b$$

We determined the reliability of the assay using human serum or plasma. The inter-assay coefficient of variation (ten identical samples derivatized separately) was 3.49 %; the intra-assay coefficient of variation (ten injections of the same derivatized sample) was 2.25 %. These coefficients of variation represent an appreciable improvement over those reported by Kage et al. (2004). We examined whether the anticoagulant used affected the plasma formate concentration, and whether plasma levels differed from serum levels. We found no difference between the formate concentrations in serum or in either EDTA or heparinized plasma (Table 1). The recovery of formate added to human serum was 102 and 106 %, respectively, from samples in which the formate concentration was increased by 25 and 50 μM (Table 1). This represents a considerable improvement over the recoveries of 65–85 % reported by Kage et al. (2004). The day-to-day reliability of the assay was determined by the successive assay for 20 days of two quality control serum samples (termed QC sera) that had been frozen in aliquots. Analysis of these sera gave formate concentrations of 33.45 ± 1.02 μM for QC1 and 39.57 ± 0.92 μM for QC2.

We addressed the effect of hemolysis on the assay. Samples with various degrees of hemolysis were prepared by spiking increasing amounts of hemolysate into serum samples from the same subject pool. Total volume was balanced across all samples by adding an appropriate amount of normal saline. This produced samples ranging from 0 to 5 % hemolysis (sufficient to produce a hemoglobin concentration of 7.5 g/L). The formate values for the hemolyzed samples were somewhat more variable than those for unhemolyzed serum. There was no significant change in serum formate at 1 or 2 % hemolysis. However, samples with between 3 and 5 % hemolysis showed a statistically significant, though small, increase in formate (Table 1). The method may also be used for the measurement of formate in urine. We found the recovery of added formate from human urine samples to be 101 and 103 %, respectively, when the formate concentrations were increased by 25 and 50 μM. In general, the urinary formate concentrations were higher than serum levels, varying from 60 to 200 μM. They were also more variable, which is not surprising given the variability in daily urine volumes.

We wish to add a cautionary note. Formate seems to be a ubiquitous contaminant, variably found in small quantities in glassware, plasticware and reagents. We advise that all batches of glassware and plasticware be checked for contamination by determining the magnitude of the intercept in a standard curve on the y-axis. The blank (intercept) value can often be reduced by rinsing glass and plasticware with high quality water and allowing them to dry before use. We urge particular caution with EDTA, since formaldehyde is used in its commercial synthesis. We analyzed formate contamination in 15 different bottles of EDTA from a variety of manufacturers. These were different preparations (sodium and potassium salts as well as the acid) obtained from different laboratories in our department. We found detectable formate in many of these EDTA preparations. For this reason, we urge particular caution when plasma is prepared in EDTA tubes. It would be best to check each batch for contamination by introducing high quality water, rather than blood, to the tubes and subsequently determining the formate concentration.

We employed the new assay to measure formate levels in sera from a cohort of 130 adults. Fresh serum samples were prepared from blood samples collected from a non-pregnant, non-fasting, adult, outpatient population, from 20 to 80 years of age. These subjects had normal complete blood counts, vitamin B12 status, and creatinine levels. All blood samples were collected in serum separator tubes and centrifuged at 3,750g for 5 min. Serum was stored at −70° until analysis. This cohort had a mean formate concentration of 30.8 μM, with a standard deviation of 6.7 μM (Fig. 4a). Further analysis of these data revealed a specific effect of sex in which formate levels in men (Fig. 4b) (32.0 ± 7.9, $n = 71$) were significantly higher than in women (Fig. 4c) (29.4 ± 4.5, $n = 59$), ($P = 0.024$). We have not carried out further studies to investigate this sexual dimorphism. As far as we are aware, this is the first

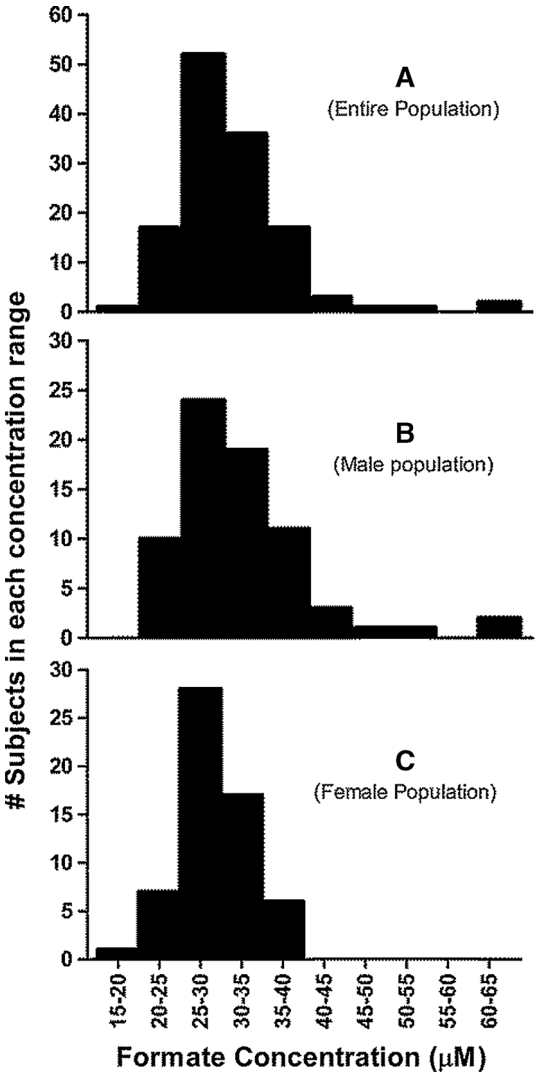

Fig. 4 Plasma formate levels in a sample of 130 adults (**a**); the formate distribution in 71 males and 59 females is shown, respectively, in (**b**) and (**c**)

report of normal serum formate levels in a human population. Finally, to further illustrate the utility of the assay, we used it to study whole body formate kinetics in the rat. We infused ^{13}C-formate at a constant rate of 9.6 μmol/h into a 180 g male, Sprague–Dawley rat, under isoflurane anesthesia; blood samples were taken every 15–20 min. Because the plasma samples contained ^{13}C-formate, ^{13}C-acetate, rather than ^{13}C-formate, was used as the internal standard. Formate concentrations were measured from the ratio of ^{12}C-formate/^{13}C-acetate (*m/z* 226 and 242). Figure 5 shows that the tracer-to-tracee (TTR) ratio of ^{13}C-formate to ^{12}C-formate reached a stable plateau value of 0.073 within 60 min. The plasma level of formate was also stable at ~65 μM. This permitted us to calculate a rate of endogenous formate production of 73 μmol/h/100 g rat, using the following equation:

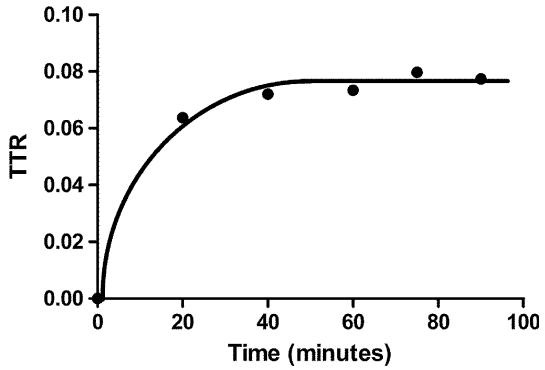

Fig. 5 Measurement of endogenous formate production in the rat. A plateau of TTR (^{13}C-formate/^{12}C-formate) is obtained within 60 min of constant infusion of ^{13}C-formate

$$R_a = \frac{F}{E_p}$$

where R_a is the rate of formate production in μmol/h, F is the infusion rate of ^{13}C-formate in μmol/h and E_p is the plateau enrichment (TTR) at steady state (Wolfe et al. 1982). We are aware of only one other study of in vivo formate kinetics; this was carried out in sheep (Annison and White 1962). They reported rates of de novo formate production ranging from 80 to 200 μmol/h/kg body weight. These results of Annison and White were obtained using ^{14}C-formate, which is unlikely to be used in human studies.

In conclusion, we have described an isotope-dilution assay for formate, which is capable of measuring the low concentrations that occur in plasma and serum. It is also suitable for use on urine samples. The assay is robust and easy to use. Unlike previous formate assays, it may be used to measure whole body formate kinetics. We have employed it to measure the kinetics of endogenous formate production in the rat as well as to define formate levels in a Newfoundland (folate-supplemented) population. We anticipate that this method will be useful for human population studies to determine how formate varies as a function of disease, diet or genetic variation. This method will also permit studies of formate kinetics in humans, avoiding the use of radioactive isotopes. Finally, the method may be used to measure formate levels in cases of suspected methanol intoxication.

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Conflict of interest None of the authors has a conflict of interest to declare.

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