Table 2. LOD, LOQ, and linearity data.

Analyte	LOD, nmol/L	LOQ, nmol/L	Slope	Intercept	R^2	$S_{y\|x}$[a]	Sa[b]	Sb[c]
5mTHF	0.07	0.13	0.037	−0.002	0.995	0.14	0.0008	0.051
HmTHF	0.20	0.40	0.026	−0.013	0.995	0.10	0.0006	0.035
FA	0.27	0.53	0.109	0.029	0.997	0.32	0.0018	0.115
5fTHF	0.52	1.03	0.132	0.063	0.997	0.40	0.0023	0.145
pABG	0.08	0.17	0.080	0.181	0.993	0.34	0.0020	0.123
apABG	0.13	0.27	0.018	0.027	0.993	0.08	0.0005	0.029

[a] Standard error of the estimate.
[b] Standard error of slope.
[c] Standard error of intercept.

(Table 3). The intra-run CVs ranged from 5.0%–9.9%, and the inter-run CVs from 3.3%–9.5%, and assay imprecision was comparable to that reported for other folate methods based on LC-MS/MS *(9, 32)*.

DISTRIBUTION OF FOLATE SPECIES

The median (range) of total folate concentration in 168 fresh serum samples from healthy Norwegian blood donors was 19.8 (7.0–174.0) nmol/L. The median (range) for 5mTHF was 16.4 (5.8–71.3) nmol/L, for hmTHF 2.3 (0.0–12.7) nmol/L, and for FA 0.0 (0.0–74.8) nmol/L; 5fTHF was not detected. This corresponds to 85.8%, 12.1%, 2.1%, and 0% of the total folate, respectively.

Table 3. Imprecision of the assay.[a]

	Intra-run		Inter-run	
	Concentration, nmol/L (n = 20)	CV, %	Concentration, nmol/L (n = 10)	CV, %
5mTHF	11.0 (0.9)	8.0	10.4 (0.4)	4.1
	31.1 (2.7)	8.9	32.4 (1.6)	5.1
	109.0 (5.9)	5.4	103.9 (4.9)	4.7
HmTHF	1.4 (0.1)	9.7	1.5 (0.1)	7.4
	4.0 (0.3)	8.1	5.6 (0.4)	7.1
	39.8 (2.5)	6.4	44.2 (2.5)	5.7
FA	1.0 (0.1)	8.4	1.1 (0.1)	11.9
	4.1 (0.3)	6.8	4.5 (0.3)	6.4
	47.5 (3.1)	6.5	45.5 (2.8)	6.1
5fTHF	1.2 (0.1)	7.6	1.0 (0.1)	10.5
	3.7 (0.3)	8.0	4.1 (0.4)	9.9
	42.1 (3.4)	8.9	38.5 (2.1)	5.5
pABG	1.3 (0.1)	7.7	1.3 (0.1)	9.6
	5.1 (0.4)	8.5	4.8 (0.2)	3.4
	41.3 (2.6)	6.3	39.6 (2.2)	5.6
apABG	1.3 (0.1)	6.4	1.0 (0.1)	8.5
	4.5 (0.2)	5.0	4.3 (0.3)	7.1
	46.2 (2.6)	5.7	40.1 (1.9)	4.8

[a] Concentrations are given as the mean (SD).

The high concentration of hmTHF in human serum was unexpected and has not previously been reported. hmTHF has been described as an oxidation product of 5mTHF *(11)*, and we have detected substantial amounts in sera stored at room temperature for weeks or at −20 °C for years. But the samples used for assay validation were stored in the dark on ice for 30–150 min before centrifugation and separation of the serum fraction, which was then stored at −80 °C for <2 years. The possible existence of hmTHF in vivo should be investigated in future studies, taking particular measures to avoid folate oxidation in vitro.

The observation that 5mTHF is the most abundant folate species in human serum confirms consistent reports by others *(9, 33)*. The concentration of FA was lower than that detected in US population (in which FA accounted for 8% of total folate) *(9)*, and only 1 of 168 sample donors had FA above 2.0 nmol/L, i.e., 74.8 nmol/L FA. This finding could be explained by the fact that there is no dietary FA fortification in Norway.

MATERIALS AND METHOD COMPARISON

We compared the total concentration of folate in 168 serum samples measured by this LC-MS/MS method with the concentrations measured by a microbiologic method and a newly developed pABG assay *(34)*. These comparisons gave a correlation of $r^2 = 0.92$ and $r^2 = 0.93$, respectively (Fig. 2). The median (range) folate concentration measured with the LC-MS/MS assay [19.8 (7.0–174.0) nmol/L] was higher than that obtained with the microbiological method [12.5 (4.6–141.6) nmol/L] and the pABG assay [17.9 (6.8–141.0) nmol/L]. The presence of hmTHF in the samples [2.3

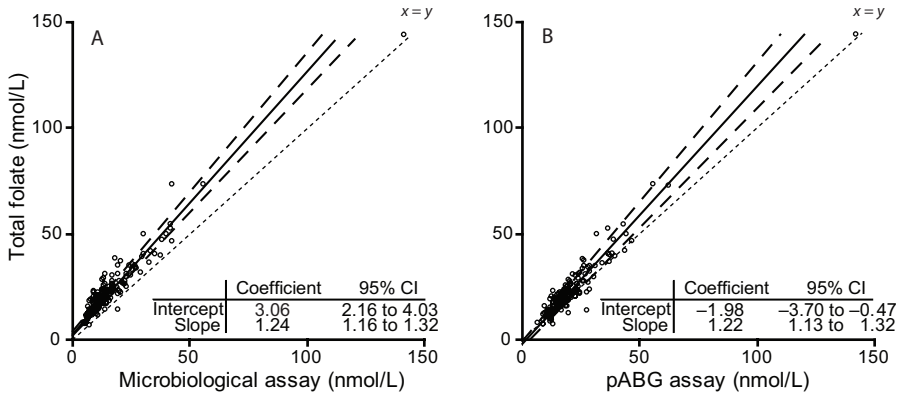

Fig. 2. Comparison of methods by Passing and Bablok regression analyses.

Total folate (the sum of 5mTHF, hmTHF, FA, and 5fTHF) measured with the current assay was compared with folate measured with the microbiologic assay (A) and the pABG assay (B). The solid line represents the regression line, dashed lines the 95% CI, and dotted line the identity line ($x = y$); n = 168.

(0.0–12.7) nmol/L] could partly account for the lower concentration obtained with the microbiological method, because hmTHF does not support the growth of *Lactobacillus rhamnosus* (formerly known as *Lactobacillus casei*) *(35)*.

FOLATE CATABOLITES

The 2 folate catabolites, pABG and apABG, have been detected in urine *(36)* and have also been reported in serum *(26)*. Our observation of low concentrations of pABG in serum from healthy blood donors (mean 0.07 nmol/L) is in disagreement with a previous report of a mean pABG concentration of 8.9 nmol/L in serum from healthy volunteers *(26)*. These results were obtained with serum samples that were acidified without any antioxidants present. This process is known to lead to partial conversion of folate to pABG *(37)*, and we confirmed the presence of similar amounts of pABG in serum subsequent to such sample preparation. Thus, the presence of about 10 nmol/L pABG in serum *(27)* seems to be an artifact related to partial conversion of folate to pABG at low pH. We also measured apABG concentrations (mean 0.47 nmol/L) lower than previously reported (mean 3.0 nmol/L) *(26)*.

We measured pABG and apABG in 39 serum samples from patients with increased serum creatinine concentrations (130–957 μmol/L). These samples had increased concentrations of the folate catabolites compared to the concentrations in healthy blood donors. The median (range) concentration of pABG was 0.3 (0.0–13.7) nmol/L and of apABG 3.5 (0.5–52.9) nmol/L. The mean total folate value in these samples was 38.9 nmol/L, which is substantially higher than in the healthy individuals. pABG and apABG showed a positive correlation with total folate (Fig. 3).

In summary, we developed an LC-MS/MS method for the determination of 5mTHF, hmTHF, FA, 5fTHF, pABG, and apABG in human serum. This method is the first reported that allows simultaneous analysis of the 4 different folate species together with

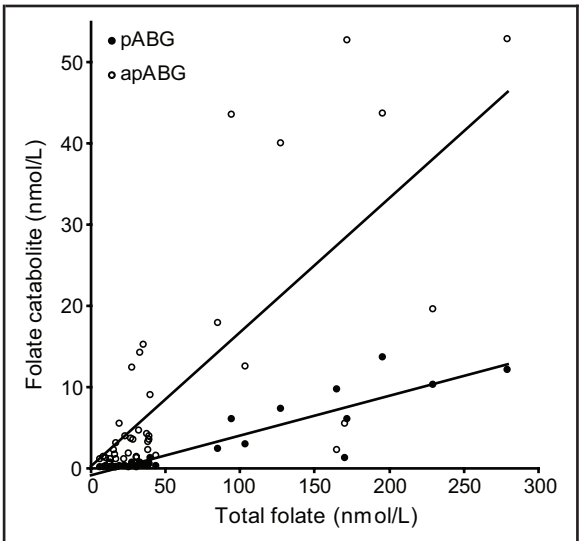

Fig. 3. Total folate, pABG, and apABG in patients with increased serum creatinine.

pABG (Spearman $R = 0.88$, $P < 0.001$) and apABG (Spearman $R = 0.77$, $P < 0.001$) showed a significant correlation with total folate; n = 39.

the folate catabolites in serum. The method also measures hmTHF in serum, which is a folate form previously believed to occur only after strong oxidation of 5mTHF *(29)*. A fast and automated sample preparation combined with short retention time of the analytes ensures a high sample throughput of 192 samples per 24 h.

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