Journal of Chromatography, 529 (1990) 155–160
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5297

Note

Analysis of blood acetate by head-space gas chromatography: comparative studies of reagents for the methyl esterification

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(First received September 12th, 1989; revised manuscript received March 5th, 1990)

Acetate (AcOH) occurs physiologically in blood and is sometimes determined as an indicator for some diseases or in hemodialysis. AcOH is a metabolite of ethanol (EtOH), and it has also been suggested that an increased level of blood AcOH during EtOH oxidation might be used as an indicator for alcoholics [1,2]. Gas chromatographic (GC) methods for AcOH are generally complicated, time-consuming and, sometimes, less accurate [3–6], because it is difficult to extract water-soluble AcOH from biological samples by organic solvent. Kveim and Bredesen [6] injected blood directly into the column, which was thus readily contaminated.

AcOH in aqueous samples can be rapidly and simply analysed as methyl acetate (AcOMe) by head-space GC [7,8]. These methods used sulphuric acid (H_2SO_4) to catalyse the methyl esterification of AcOH.

This paper describes the use of other esterification reagents in the analysis of physiological levels of blood AcOH.

EXPERIMENTAL

Analysis of AcOH by head-space GC

All reagents were of analytical grade from Wako (Osaka, Japan). An aliquot of catalyst for methyl esterification and/or methanol (MeOH) was added to

0378-4347/90/$03.50

0.5 ml of the samples in glass head-space vials (16 ml), as described below. After the addition of 0.05 ml of the internal standard [I.S., 1% (v/v) acetonitrile], each vial was sealed with a rubber septum and an aluminium cap. Samples were deproteinized with MeOH. The vials were incubated at 60 °C for 30 min, and then 1 ml of the gas phase was injected into the gas chromatograph. The calibration curves were obtained with aqueous solution and human whole blood containing appropriate concentrations (0.025–10.0 m*M*) of sodium acetate (AcONa) and the curves for blood AcOH were corrected for the physiological level of blood AcOH (0.048 m*M*) calculated by the calibration curves in aqueous solution. The effects of added amounts of catalysts and incubation time on the methyl esterification of 6 m*M* AcONa were examined by comparison with 6 m*M* standard AcOMe.

Methyl esterification

Five different reagents were used: H_2SO_4, hydrochloric acid (HCl), methyl iodide (MeI), boron trifluoride (BF_3) and boron trichloride (BCl_3).

In the H_2SO_4 method, 0.1 ml of concentrated H_2SO_4 was added to the samples. The vials were cooled to room temperature. Then 0.5 ml of 99% MeOH and 0.05 ml of I.S. were added, and the vials were incubated as described above.

In the HCl method, 0.1 ml of HCl and 0.5 ml of 99% MeOH were added to the samples.

In the MeI method, 0.1 ml of 10 *M* sodium hydroxide and 0.01 ml of MeI were added to the samples.

In the BF_3 method, 0.5 ml of 13.5–14.5% BF_3–MeOH complex was added to the samples.

In the BCl_3 method, after the addition of 0.5 ml of 99% MeOH, 0.2 ml of 99.999% BCl_3 cooled at −80 °C was added to the samples.

GC conditions

A Shimadzu GC-7AG gas chromatograph with a flame ionization detector and a packed column (1 m × 2.6 mm I.D., 25% PEG 6000 on Shimalite, 80–100 mesh, Shimadzu, Kyoto, Japan) was used. The temperatures of the injection port and the oven were 140 and 90 °C, respectively. The carrier gas was nitrogen and the flow-rate was 40 ml/min. The peak-area ratios of AcOMe and I.S. were used for the quantitations.

Effect of EtOH on the esterification

After the addition of 0.02 ml of EtOH and 6 m*M* AcONa to 0.5 ml of blood, the sample was analysed by the BF_3 method. Peak- area ratios of ethyl acetate (AcOEt) and AcOMe were compared with each other to investigate the interference by blood EtOH.

RESULTS AND DISCUSSION

When a column packed with PEG 6000 was used, the AcOMe peak appeared before the large peak of MeOH, and the last peak (I.S.) appeared within 3 min (Fig. 1). When a column with Porapak Q or Porapak QS was used as reported previously [7,8], the AcOMe peak appeared in 6 or 16 min, after the large peak of MeOH. This increased retention resulted in a broader peak and lower accuracy, and small amounts of AcOH could not be quantified owing to tailing of the MeOH peak. Therefore, AcOH can be analysed more rapidly and accurately with the use of PEG 6000.

In each method another peak was observed at 0.25 min (Fig. 1). When H_2SO_4 or HCl was used as a catalyst, the peak area increased with increasing incubation time or amount of added catalyst, suggesting the formation of a by-product (Table I). In particular, the excessive increase of the by-product in the HCl method seemed to cause a compensatory decrease of the AcOMe peak, insignificantly. Furthermore, HCl gas might be generated which would corrode the column. It was thus concluded that the HCl method was unsuitable for the determination.

Though the BF_3 method showed the same peak (Fig. 1), the substance was not a by-product but an original component of the BF_3–MeOH complex: the peak-area ratio did not change with the incubation time or the amount of catalyst. When BCl_3 was used, however, the peak of the by-product appeared and increased with the incubation time. Because the boiling point of BCl_3 was too low (12.4°C) to treat at room temperature, and because the mixture emitted intense heat, the BCl_3 method was also unsuitable for the rapid and simple analysis of AcOH.

MeI was useless for head-space GC, since the large peak of MeI overlapped the peak of AcOMe. Consequently, the BF_3 method was investigated in this study and compared with the H_2SO_4 method reported previously [7,8].

The physiological levels of blood AcOH were 0.048 and 0.062 m*M*, as measured by the BF_3 and H_2SO_4 methods, respectively. Only 0.1 ml of H_2SO_4 was required for methylation of AcOH, whereas 0.5 ml of BF_3–MeOH complex was required for quantitative esterification (Table I), indicating the high reactivity

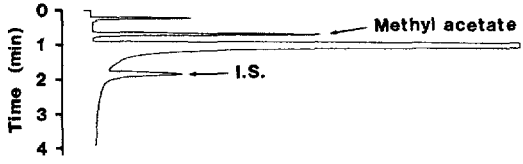

Fig. 1. Gas chromatogram of AcOMe as I.S. [1% (v/v) acetonitrile]. The gas phase of a head-space vial containing 6 m*M* AcOH, 0.5 ml of BF_3–MeOH complex and 0.05 ml of I.S. was injected after incubation at 60°C for 30 min.

TABLE I

EFFECT OF INCUBATION TIME AND AMOUNTS OF CATALYST ON THE FORMATION OF AcOMe AND BY-PRODUCT

Amount of added catalyst (ml)	Percentage of AcOMe[a] and by-product[b]				
	10 min	20 min	30 min	40 min	60 min
H_2SO_4					
0 1	95.6 ±16.3	91.2± 16.1	86.7± 9.36	–	–
	(4.88± 0.87)	(11.9± 2.63)	(17.5± 2.92)		
0.2	96.0 ± 1.38	85.4± 0.77	90.1± 6.13	–	–
	(13.2 ± 5.98)	(27 0± 0.78)	(56.2± 8.04)		
0.5	92.9 ±10.1	100.4± 16.9	104.1± 16.8	–	–
	(43.2 ± 4.17)	(121.4± 13.1[c])	(158.5±118.5[c])		
HCl					
0.1	87.5 ± 4.58	87.1± 10 4	81.1± 9.03	–	–
	(15.0 ± 1.65)	(35.1± 5.61)	(51.0± 8.80)		
0 2	101 6 ± 7.09	98.0± 1.40	96.2± 4.37	–	–
	(68.2 ± 8.37)	(170.8± 18.4[d])	(254.1± 27 0[d])		
0.5	106.4 ± 3.99	101.9± 5 46	98 9± 8.79	–	–
	(280.2 ±21.8)	(811.0±135.7[c])	(1084.8±106.8[d])		
BF_3					
0.1	14 8 ± 2.95	19.0± 2.95	–	38.2±21.1[c]	55.7± 41.7[d]
0.2	38.6 ± 4.12	51.8± 5.21	–	84.2±16.3[c]	99.9±121 8[c]
0.5	86 9 ±23.8	95.8± 27.7	–	105.0±55.4[c]	105.7± 49.2[c]
BCl_3					
0.2	81.6 ±28.0	91.0± 15.6	94.4± 14.2	–	–
	(53.3 ±10.1)	(93.7± 19.3[c])	(120.9± 39.5[d])		

[a]Mean ± S.D. for six determinations: the level of the same amount of standard AcOMe designated as 100%.
[b]Mean ± S.D of peak-area ratio of by-product versus I.S in parentheses.
[c]$p < 0.05$.
[d]$p < 0.01$, compared with the levels at 10 min by Student's t-test.

of H_2SO_4. However, the calibration curve obtained in the H_2SO_4 method was not linear, and the Bartlett's test [9] showed a heterogeneity of variance (Fig. 2 and Table II), suggesting that the procedure was not reproducible. The calibration curve by this method for the aqueous solution was, similarly, not linear ($x = 0.64y^2 + 0.69y - 0.49$). Generally, when alkyl esterification was catalysed by H_2SO_4, by-products such as alkyl hydrogen sulphate and diethyl ether might be produced in vials at the same time [10]. Thus, it was considered that the catalytic action of H_2SO_4 was unreliable.

In contrast, the BF_3 method gave a linear calibration curve and a high correlation coefficient (Fig. 2), and the Bartlett's test showed homogeneity of variance (Table II). Similarly, a linear calibration curve ($x = 4.33y - 0.28$, $r = 0.99898$) was obtained by the same method in aqueous solution. From these

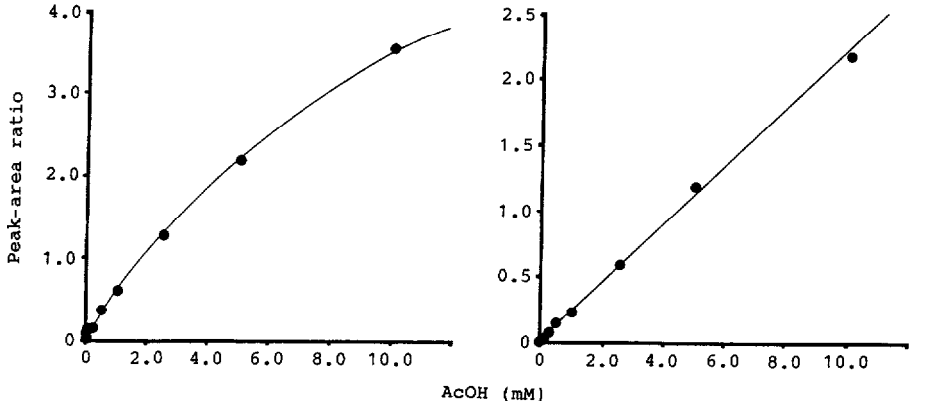

Fig. 2. Calibration curves for the determination of AcOH in whole blood obtained by both the H_2SO_4 method (left: $x=0.39y^2+1.47y-0.08$, $r=0.97862$) and the BF_3 method (right: $x=4.57x-0.13$, $r=0.99320$). Each point of the curves represents the average of six determinations.

TABLE II

ACCURACY OF ACETATE DETERMINATION

Values in parentheses are coefficients of variation (%).

AcOH (m*M*)	Peak-area ratio (mean ± S.D., $n=6$)	
	H_2SO_4	BF_3
0.025	0.375 ± 0.051 (10 53)	0.066 ± 0.008 (12.04)
0.050	0.581 ± 0.041 (8.03)	0.071 ± 0.010 (12.92)
0.10	0.654 ± 0.050 (9.15)	0.081 ± 0.009 (9.84)
0.25	0.620 ± 0.037 (5.72)	0.123 ± 0.008 (6.22)
0.50	0.847 ± 0.070 (8.73)	0.202 ± 0.011 (6.04)
1.00	1.030 ± 0.065 (6.10)	0.289 ± 0.029 (9.80)
2.50	1.656 ± 0.123 (7 35)	0.657 ± 0.047 (7.35)
5.00	2.422 ± 0.060 (2.45)	1.272 ± 0.118 (9.68)
10.00	3.514 ± 0.170 (4.79)	2.283 ± 0.211 (8.87)
Bartlett's test		
χ^2	20.37 ($p<0.01$) (heterogeneity)	7.70 ($p>0.05$) (homogeneity)

results, the BF_3 method was considered to be sufficiently reproducible, and the AcOH level measured by this method reliable. BF_3–MeOH formed no by-product and was less hazardous than H_2SO_4. Therefore, it was concluded that BF_3 was more suitable as a catalyst for the methyl esterification of AcOH.

Giles et al. [8] analysed both AcOH and EtOH in blood at the same time. However, in their method, the EtOH peak appeared immediately after the large

TABLE III

ETHYL ESTERIFICATION OF ACETATE BY BLOOD ETHANOL

The sample blood contained 6 m*M* AcOH and 4% EtOH. Values represent mean ± S.D. for three determinations.

Incubation time (min)	AcOEt (peak-area ratio)	AcOMe (peak-area ratio)	Ratio[a] (%)
10	0.84 ± 0.02	23.06 ± 3.58	3.62
20	0.94 ± 0.18	25.12 ± 3.97	3.74
30	0.88 ± 0.28	25.51 ± 6.57	3.47

[a]Content of AcOMe as 100%.

peak of MeOH so that the EtOH could not be determined accurately. EtOH and AcOH should be determined separately. When any other alcohol was present in the samples, AcOH would be esterified by the alcohol to some extent. In order to investigate the interference by blood EtOH with the reaction, blood containing 6 m*M* AcOH and 4% EtOH ($\simeq$ 30 mg/ml) was analysed by the BF_3 method. This EtOH level was much higher than the normal blood EtOH level brought about by drinking (less than 4 mg/ml). The peak area of AcOEt produced by the esterification of AcOH with added EtOH was 3–4% of that of AcOMe (Table III), probably owing to the large amount of MeOH complex added (0.5 ml). Consequently, the interference by any other alcohol present in blood with the determination of blood AcOH would not be significant.

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