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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FORMATE AS BENZIMIDAZOLE IN BIOLOGICAL SAMPLES

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SUMMARY

Formate was determined as benzimidazole by high-performance liquid chromatography after reaction with *o*-phenylenediamine at 130°C for 2 h in 1 *M* perchloric acid The useful concentration range was 1.6–40 μmol/l and the determination limit was 20 pmol. The recoveries from rat liver homogenate and human urine were 90.3 ± 2.9 and 89.4 ± 2.5%, respectively Using this method, the activity of formaldehyde dehydrogenase in biological samples could be measured, and also the formate concentration in the liver and urine of rats to which methanol had been administered.

INTRODUCTION

Methylglyoxal (MG) has been reported to originate from triose, acetoacetate, acetone and L-threonine and to be metabolized to pyruvate through several routes present in microorganisms, plants and animals [1–6]. Since the biosynthesis and metabolism of MG remain to be solved, we have undertaken studies in this area. Formaldehyde dehydrogenase (FADH) (E.C. 1.2.1.1) has been purified from many organisms, including humans [7–12]. However, we think that liver does not metabolize much of the substrate, formaldehyde, formed by degradation of dimethylglycine to glycine and by demethylation reactions of O–CH_3, N–CH_3 and S–CH_3 in natural products and chemical drugs. If FADH is not present in animal tissues, formaldehyde is also metabolized via the folate pathway. During the study of the MG metabolism, we assumed that FADH catalyses the conversion of MG to pyruvate rather than formaldehyde to formate. In order to explain the reason for the existence of FADH, we first aimed at isolating it from rat liver. However, we experienced a difficulty in measuring the enzyme activity at 340 nm for NADH

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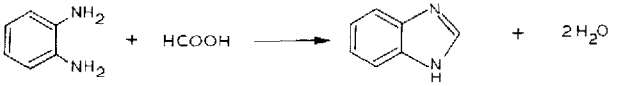
in the case of rat liver homogenate or the crude enzyme, because of the low sensitivity. For this reason, we decided to determine formate sensitively for the assay of FADH. We first applied the gas chromatographic method to the determination of formate as the pentafluorobenzyl ester [13]. In spite of our efforts, we failed in determining formate in biological samples because of the instability of the ester, but other fatty acids could be determined by this method.

Enzymatic methods [14,15] were also available for the determination, but were found to be unsuitable for the crude enzyme because of underestimation and low sensitivity. Subsequently, we tried to develop a highly sensitive high-performance liquid chromatographic (HPLC) method. Formate was reacted with *o*-phenylenediamine (*o*-PDA) and the benzimidazole formed was extracted and measured at 267 nm by HPLC.



Benzimidazole is chemically very stable against acids, alkalis and oxidizing agents and has a high molar absorption coefficient of $6.78 \cdot 10^6$ cm^2 mol^{-1} at 267 nm in the mobile phase. This method was applicable to both urine samples and liver homogenates. In this paper, investigation of methanol metabolism in rats and mice is also presented as another example of the application of the method developed.

EXPERIMENTAL

Chemicals

o-PDA was purchased from Wako (Osaka, Japan) and purified by sublimation at 100°C under reduced pressure (2.5 mmHg). The crystals were stored over silica gel in a refrigerator. The sublimation is indispensable for diminishing the blank peak observed in HPLC. NAD was obtained from Oriental Yeast (Osaka, Japan). Glutathione (GSH) was kindly supplied by Yamanouchi Pharmaceutical (Tokyo, Japan). Formate dehydrogenase and *p*-iodonitrotetrazolium violet were purchased from Sigma (St. Louis, MO, U.S.A.), 3,4-dichloroacetophenone, 2,4-dichloroacetophenone and 4-chloro-1,2-phenylenediamine from Tokyo Kasei Kogyo (Tokyo, Japan) and 2,5-dichloroacetophenone, 4,5-dimethyl-1,2-phenylenediamine and 4,5-dichloro-1,2-phenylenediamine from Aldrich (Milwaukee, WI, U.S.A.). 4-Trifluoromethyl-1,2-phenylenediamine was prepared in our laboratory [16]. 5,6-Dimethyl-, 5-trifluoromethyl-, 5,6-dichloro- and 5-chlorobenzimidazole were synthesized by reaction of sodium formate and the corresponding *o*-PDA at 120°C in 2 *M* hydrochloric acid for 2 h. Sodium formate and all other reagents of analytical-reagent grade were purchased from Katayama Chemical (Osaka, Japan).

Instruments

A Model 572 liquid chromatograph (Gaschro-Kogyo, Osaka, Japan) equipped with a Model 502 variable-wavelength detector (Gaschro-Kogyo) was used. Benz-

imidazole was determined using a 150×6 mm I.D. Unisil NQ C_{18} (5 μm) column (Gaschro-Kogyo) with isocratic elution with a mixture of 10 m*M* KH_2PO_4–H_3PO_4 (pH 2.1) and acetonitrile (80:20, v/v) and monitored at 267 nm. The chromatograph was operated at 1 ml/min and 25°C. Spectrophotometric measurements were performed with a Shimadzu (Kyoto, Japan) UV-180 double-beam spectrometer.

Formate in water

Aqueous solutions were prepared at concentrations of 5.4 and 27 μg/ml for sodium formate and 0.6 and 2.8 mg/ml for *o*-PDA. Each aqueous solution of formate and *o*-PDA (10–50 μl) was placed in a 5-ml vial with a tight cap. Perchloric acid (1 *M*) was added to the vial to make a final volume of 0.5 ml and the mixture was heated at 130°C for 2 h. The reaction mixture was neutralized with 0.25 ml of 1 *M* sodium carbonate solution and extracted twice with 1 ml of ethyl acetate–ethanol (9:1, v/v). The organic layer was transferred to another vial and evaporated in vacuo at room temperature using a Savant Speed Vac Concentrator (Model SVC-100 H) (Savant Instruments, New York, U.S.A.). The residue was dissolved in 0.5 ml of the mobile phase described above and a 20-μl sample was analysed by HPLC.

Formate in urine

A urine sample (0.3 ml) was transferred to a Dowex 50W-X2 (H^+) (100–200 mesh) column (7.0×0.9 cm I.D.), which was washed with 10 ml of water. The unadsorbed portion and the washings were successively applied to a Dowex 1-X2 (OH^-) (100–200 mesh) column (7.0×0.9 cm I.D.), which was washed with 10 ml of water. After formate had been eluted with 20 ml of 0.25 *M* sulphuric acid the eluate was made weakly alkaline with 0.25 *M* barium hydroxide solution and centrifuged at 1600 *g* for 10 min. The supernatant was evaporated under reduced pressure and the residue was dissolved in 0.6 ml of water, 20 μl of which were reacted with 20 μl of 75 m*M* *o*-PDA and 0.46 ml of 1 *M* perchloric acid in the vial at 130°C for 2 h. After reaction, benzimidazole was extracted and analysed by HPLC as described above.

Formate in rat liver homogenate

A 2-ml volume of 0.5 *M* perchloric acid was added to 50 μl of the rat liver 700 *g* homogenate. After centrifugation, a 20-μl sample of the supernatant was converted into benzimidazole and treated as described under *Formate in urine*.

Administration of methanol

Male Wistar strain albino rats aged 4 weeks (90–100 g) and male DDY strain mice (about 20 g) were divided into two groups each. One group was injected intraperitoneally with a 30% (w/v) methanol solution in physiological saline (6 g/kg body weight) and another group with saline. Each group was maintained on normal solid food and water ad libitum for 2 days. Urine samples for the term were collected in the case of rats and determined for formate. The animals were decapitated after 2 days and the livers were removed immediately and used for the determination of FADH activity and formate.

Measurement of formaldehyde dehydrogenase activity in liver

Enzyme source. Fresh rat liver was perfused with physiological saline and homogenized in 3 volumes of 50 m*M* potassium phosphate buffer (pH 7.4) containing 5 m*M* 2-mercaptoethanol using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 700 *g* for 15 min.

Incubation conditions. The incubation was carried out according to a modification of the procedure of Uotila and Koivusalo [17]. The incubation mixture of 0.9 ml contained 10 μl of 0.1 *M* formaldehyde, 20 μl of 0.05 *M* GSH, 20 μl of 0.04 *M* NAD, 20 μl of 0.8 *M* nicotinamide, 50 μl of enzyme and 0.12 *M* sodium phosphate buffer (pH 8.0). The reactions were started by addition of formaldehyde and incubated at 25°C for 10 min. To the reaction mixture 0.1 ml of 20 m*M* sodium borohydride was added and reacted at 0°C for 30 min. After addition of 1 ml of 1 *M* perchloric acid to the reaction mixture, it was centrifuged at 1600 *g* for 10 min. A 20-μl aliquot from the supernatant was analysed for formate. The enzyme activity is expressed in international units.

Protein determination

Protein concentrations were determined by means of the biuret reaction [18].

Administration of sodium formate to rats

The rats described above were injected intraperitoneally with various amounts of sodium formate (25, 50, 75, 100, 125, 150 and 200 mg) in 2.5 ml of physiological saline. The rats were maintained on normal solid food and water for 45 h. Each urine sample was collected for the term. After decapitation, the livers were removed and analysed for formate.

RESULTS

Chromatographic profile of benzimidazole

Fig. 1 shows the elution profiles obtained from authentic benzimidazole (a), when sodium formate was reacted with *o*-PDA (b) and the perchloric acid extract of rat liver was reacted with *o*-PDA (c). The analysis time of 5.4 min is satisfactory, but the shape of the peaks is unsatisfactory. When the benzimidazole was analysed using 10 m*M* KH_2PO_4 (pH 2.1)–acetonitrile (95:5, v/v) on a Unisil ODS QT-SK (5 μm) column (150×4.6 mm I.D.) and the chromatograph was operated at 1 ml/min and 40°C, the shape and separation of the benzimidazole peak were perfect. However, in this instance, the peaks at 12.7 and 15 min in Fig. 1b and c were not eluted and hence the column had to be washed. This was impractical, so we adopted the mobile phase described under Experimental.

Reaction conditions and extraction of benzimidazole

The effects of the reaction time and temperature on the formation of benzimidazole were investigated at 20, 100, 120, 130, 140 and 150°C for up to 5 h in 1 *M* perchloric acid. Benzimidazole was not formed at 20°C and was obtained in the maximal yield at 130°C in 2 h. Therefore, 2 h at 130°C was chosen for the present method, giving a yield of 89.3 ± 1.0%. The effect of the molar ratio of *o*-

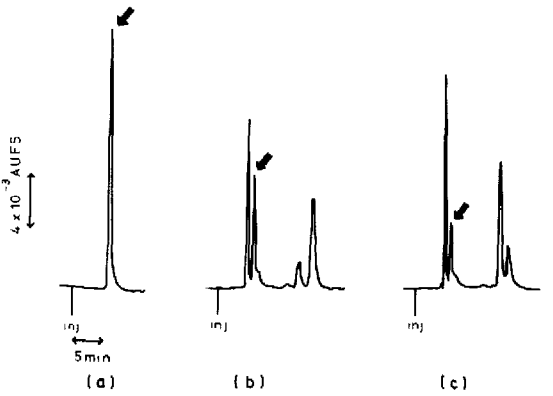


Fig. 1 High-performance liquid chromatograms. (a) Benzimidazole (40 nmol) was dissolved in 0.5 ml of the mobile phase, 20 μl of which were injected; (b) sodium formate (20 nmol) was reacted with *o*-PDA in 1 *M* perchloric acid at 130°C for 2 h and benzimidazole formed was extracted and treated as described under *Formate in water*; (c) the rat liver homogenate was treated as described under *Formate in rat liver homogenate* and the benzimidazole formed was extracted and injected.

PDA to formate on the yield of benzimidazole was maximal at a 60:1 ratio. The reaction yields were 42.4 ± 7.3, 87.2 ± 4.9, 90.6 ± 2.5 and $77.4 \pm 5.9\%$ in 0.1, 0.5, 1.0 and 2.0 *M* perchloric acid, respectively. Benzimidazole was extracted with several organic solvents, the best results being obtained with ethyl acetate–ethanol (9:1, v/v). When various amounts of benzimidazole dissolved in 0.5 ml of the mobile phase were extracted twice with 1 ml of the mixture, $93.2 \pm 3.9\%$ of benzimidazole was recovered.

Standard and calibration graphs

For the standard graph, various amounts of benzimidazole were dissolved in the mobile phase, 20 μl of which were analysed by HPLC. Various amounts of formate were reacted with *o*-PDA at a molar ratio of 60:1 with respect to formate in 0.5 ml of 1 *M* perchloric acid at 130°C for 2 h. After neutralization with 1 *M* sodium carbonate solution and extraction with ethyl acetate–ethanol (9:1, v/v), the organic layers were evaporated under reduced pressure. The dried residues were dissolved in 0.5 ml of the mobile phase, 20 μl of which were analysed by HPLC.

The standard and calibration graphs were linear from 0.8 nmol per 0.5 ml to at least 20 nmol per 0.5 ml. The detection limit was 5 pmol at a signal-to-noise ratio of 2:1.

Recovery tests

Various amounts of formate were added to rat liver homogenate and to normal human urine and their recoveries were measured by each procedure as described above. The results are summarized in Tables I and II, which show recoveries of 90.3 ± 2.9 and $89.4 \pm 2.5\%$, respectively.

Comparison of results obtained by the present method and the enzymatic method

Urine samples and liver homogenate from rats injected with formate were analysed for formate by the present method and the enzymatic method [15]. These

TABLE I

RECOVERY OF FORMATE ADDED TO RAT LIVER SUPERNATANT

Rat liver (1 g) was homogenized with 3 ml of 50 m*M* phosphate buffer (pH 7.4), the homogenate was centrifuged at 700 *g* for 15 min and to 50 μl of the supernatant various amounts of sodium formate were added. After the superatant had been deproteinized with 1 *M* perchloric acid, *o*-PDA was added to the supernatant and reacted at 130 °C for 2 h. The benzimidazole formed was determined by HPLC.

Amount added to 50 μl of extract of liver (mg)	Determined value (mg per 50 μl of extract of liver)	Recovery (%)
None	0.13	
0.05	0.18	91 1
0.10	0.22	92.3
0.15	0.26	88 7
0.20	0.31	90.6
0 25	0.36	91.5
0.50	0.58	90.2
0 75	0.81	91.0
1.00	1.02	89.2
1 25	1.23	88.1
Mean ± S.D		90.3 ± 2.9

results are shown in Fig. 2, indicating that both methods give essentially the same values and a correlation coefficient of 0.998 for rat urine and 0.968 for rat liver. However, it should be noted that both methods were carried out in a range of

TABLE II

RECOVERY OF FORMATE ADDED TO HUMAN URINE

After various amounts of sodium formate had been added to 0 3 ml of normal human urine, the samples were successively applied to Dowex 50-X2 and Dowex 1-X2 columns. The sulphuric acid eluate was neutralized with barium hydroxide and centrifuged. The supernatant was evaporated to dryness, the residue was dissolved in 0.6 ml of water and 20 μl of the solution were reacted with *o*-PDA in 1 *M* perchloric acid. Benzimidazole was treated as described under *Formate in urine*.

Amount added to 0.3 ml of urine (mg)	Determined value (mg per 0.3 ml of urine)	Recovery (%)
None	0.06	
0.2	0.23	87.2
0.5	0.52	92.0
1.0	0.98	91.7
1.5	1.35	85.8
3.0	2.76	90.1
Mean ± S.D.		89.4 ± 2.5

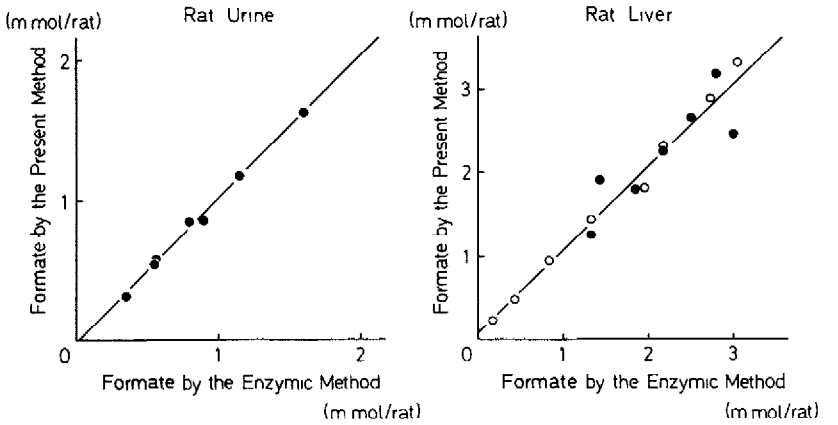


Fig. 2. Relationship between concentration of formate in rat urine and liver measured by the enzymatic method and the present method. Closed circles indicate samples from rats administered sodium formate and open circles represent 700 g of normal liver homogenate to which sodium formate was added. Urine: $y = 1\ 04x - 0.03$; $r = 0.998$. Liver: $y = 0.99x + 0.10$, $r = 0.968$.

relatively high concentrations of formate, because the detection limit for the enzymatic method was 12 mg/l [15].

Administration of methanol and formate to rats and mice

As shown in Table III, a one-dose injection of methanol (6 g/kg) resulted in a significant increase in FADH activity to 2.5 times the control for rat livers and 3.8 times the control for mouse livers after 45 h. The formate levels in liver of both animals were also elevated 45 h after the injection. About 25% of the injected formate was recovered from the rat urine samples within 45 h and 16.9 ± 4.5% of it was recovered in rat liver at 45 h after the injection (Table III). In spite of the

TABLE III

FORMALDEHYDE DEHYDROGENASE ACTIVITY IN LIVER AND FORMATE IN URINE

One group of animals was injected intraperitoneally with 30% methanol in physiological saline (6 g methanol per kg body weight). After 45 h, the animals were decapitated and the livers were removed. Liver was homogenized in phosphate buffer and the homogenate was centrifuged. The supernatant was tested for FADH activity and analysed for formate. Urine samples were collected from rats and analysed for formate. Results are means ± S.D. for five determinations.

Animal	Formaldehyde dehydrogenase activity in liver ($U \times 10^{-3}$/mg)	Total formate (mg)	
		Liver	Urine
Rat			
Control	90 3 ± 10.8	47.0 ± 3.08	11.7 ± 2.07
Methanol-treated	228 ± 15.8	65.1 ± 3.97	61.0 ± 4.46
Mouse			
Control	44 8 ± 20.2	1.78 ± 0.93	
Methanol-treated	174 ± 31.1	6.81 ± 1.94	

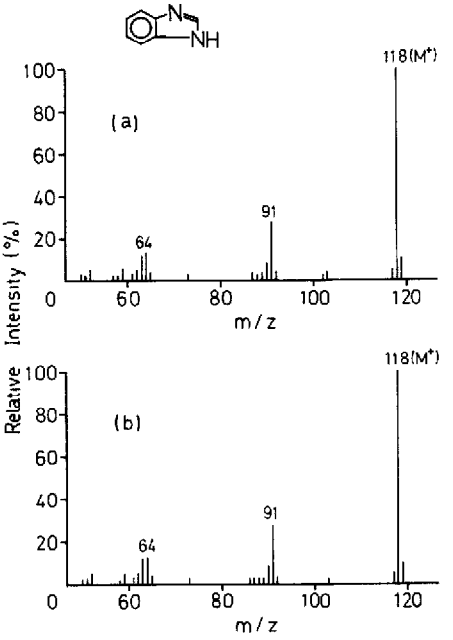


Fig. 3. Mass spectra of benzimidazole. (a) Authentic benzimidazole, (b) the reaction product of formate in urine samples from rat injected with methanol. Urine samples were treated as described under *Formate in urine* and benzimidazole was extracted and subjected to mass spectrometry.

difficulty of the complete collection of the rat urine samples, considerable amounts of formate were excreted. These values, calculated by subtracting control values, indicate that formate must be the end-product of methanol or formaldehyde in the rat.

Identification of the peak of benzimidazole

In order to identify the peak of benzimidazole, urine samples from rats injected with methanol were treated as described under *Formate in urine* and analysed by HPLC. The peak corresponding to benzimidazole was collected. This fractionation procedure was repeated about ten times. The combined fractions were evaporated under reduced pressure, the residue was dissolved in 1 ml of the mobile phase and 20 μl were analysed by HPLC. The remaining sample was dried under reduced pressure and the residue was dissolved in 1 ml of water. After extraction with two 2-ml volumes of ethyl acetate–ethanol (9:1, v/v), the organic layer was evaporated to dryness under reduced pressure. The residue was analysed by mass spectrometry (Fig. 3); the parent peak (M^+) was at *m/z* 118.

Formaldehyde dehydrogenase activity in biological samples

The proposed method was applied to the determination of formate in biological samples of homogenate level, where the enzymatic method based on NADH formation was inadequate because of the low sensitivity and underestimation. We measured the FADH activity in the livers of several mammals; the livers of cattle,

pig, rabbit, rat, hamster and mouse contain 19.32, 0.78, 0.24, 0.23, 0.13 and 0.03 units/mg, respectively. In the rat, the liver had the highest activity, followed by kidney, lung and muscle. The activity at the subcellular level of rat liver was the highest (85.1%) in the cytosolic, 6.8% in the mitochondrial and 6.9% in the microsomal fraction relative to 100% activity in the homogenate.

DISCUSSION

Although formic acid is a simple substance, its determination in body fluids and tissues is difficult. Early methods, including manometric, spectrophotometric and biological methods, were reviewed in ref. 19. However, these methods are of little utility because of their inadequate accuracy and sensitivity. Hence, the formic acid levels in biological samples given in the literature and obtained using these methods are doubtful. Subsequently, many methods for this determination have been reported but most of them are of low sensitivity and not applicable to biological samples. Methods using bacterial formate dehydrogenase [14,15] are more suitable, but 200 times less sensitive than the present method. Another method using formyltetrahydrofolate synthetase [20] is not a practical method because the enzyme is not commercially available.

After a failure in applying the gas chromatographic (GC) method reported by Chauhan and Darbre [13], we tried to modify another GC method of Chauhan and Darbre [21] using phenacyl bromide. We synthesized 3′,4′-dichlorophenacyl, 2′,4′-dichlorophenacyl and 2′,5′-dichlorophenacyl bromide by bromination of the corresponding acetophenones. Formate was then esterified and the esters were determined by GC with electron-capture detection. The results were also unsatisfactory, although acetate could be determined. As formic acid is approximately as strong as hydrofluoric acid, the esters of formate appear to be easily hydrolysed. A stable ester such as methyl formate is inadequate for sensitive determination [22].

Next, 5,6-dichloro-, 5-chloro- and 5-trifluoromethylbenzimidazole were synthesized and tested by GC with electron-capture detection. 5-Chloro- and 5-trifluoromethylbenzimidazoles were insensitive and 5,6-dichlorobenzimidazole was sensitive at levels as low as 32 pmol, but in any event the GC method was less effective than the present method using HPLC.

The biological toxicology of methanol has been studied and reviewed [23–27]. Eells et al. [28] reported that administration of methanol to rats did not produce accumulation of formate in the blood. As shown in this work, however, formate accumulated in rat liver even after 45 h. As an exceptional case, a patient poisoned with methanol excreted about 1.9 g/l of formate [26], and considerable amounts of formate appear to be excreted in normal human urine. We have found that FADH activity in rat liver cytosol was separated into three fractions by DEAE-cellulose column chromatography, and the main peak showed a larger value of V_{max}/K_m and a smaller K_m for MG than that of formaldehyde (V_{max} = maximal velocity; K_m = Michaelis constant). The physiological role of FADH in rat liver is under study.

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