Cobas Mira™ S Endpoint Enzymatic Assay for Plasma Formate*

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Abstract

In methanol intoxication, increased levels of the metabolite formate are associated with metabolic acidosis and an increased risk for ocular and neurological dysfunction. A simple method for plasma formate measurement by adaptation of a manual enzymatic assay to a Cobas Mira S analyzer is presented. Six microliters of sample is incubated for 5 min with buffer containing nicotinamide-adenine dinucleotide. Fifteen microliters of a suspension of formate dehydrogenase is then added. Absorbance at 340 nm is measured every 25 s. The NADH produced when formate is oxidized is stoichiometric to the amount of formate. The method is sensitive, reproducible, and specific and has a broad measurement range. The frozen reagents are stable for at least six months, so the described method can be applied to irregular and semi-urgent requests. A recent case is reported.

Introduction

Toxicity of methanol results from its oxidation to the highly toxic formate that is the main contributor to the metabolic acidosis (expected for formate levels above 200 mg/L), the visual disturbances, coma, or death related to methanol poisoning (1–6). Blood formate concentrations correlate well with the severity of methanol poisoning, formate levels above 500 mg/L possibly leading to permanent visual or neurological sequelae. No such correlation was found for the blood methanol concentration (1,7,8).

As a more direct measure of toxicity and to improve a poisoned patient's assessment, determination of formate in blood should be included in the prognostic elaboration, especially in those cases where the time between ingestion and treatment is unknown (2,4,9). Proposed chromatographic, colorimetric, or enzymatic procedures are complex, use reagents with short time stability, and/or lack automation (9–14).

The following report describes a simple and specific method for plasma formate measurements by adaptation of a manual enzymatic assay to an automated Cobas Mira S analyzer. Assessed reagent stability largely exceeded the manufacturer's specifications.

Materials and Methods

Apparatus

Formate concentrations were determined on a Cobas Mira S multiparametric analyzer from Roche Diagnostics Division (Basle, Switzerland).

Reagents

An enzymatic assay initially intended for bioanalysis of food was purchased from Boehringer Mannheim (Mannheim, Germany; catalog no. 979 732).

Formate dehydrogenase (FDH). Approximately 80 U were dissolved in 2.0 mL distillated water. Aliquots of 200 µL were stored at +4°C and frozen at –20°C to test reagent stability.

Nicotinamide-adenine dinucleotide (NAD), Li salt. Four hundred twenty milligrams was dissolved in 22 mL potassium phosphate buffer (pH 7.5) and 28 mL distillated water. Five-milliliter aliquots were stored at +4°C and frozen at –20°C to test reagent stability.

Standard solutions

Formate standard, 10 g/L. Formate (2.5 g, 98-100% formic acid GR, Merck, Darmstadt, Germany) was diluted to 250 mL with water filtered through a Milli-Q® Water Purification System (Millipore Corp., Bedford, MA). This solution was stable for at least 6 months at +4°C. Aqueous working solutions of 200 mg/L formate were prepared and aliquotted in 0.5-mL portions (stable for at least 6 months at –20°C).

Other chemical standard solutions included methanol, ethanol, ethylene glycol, acetone, and formaldehyde, all of which were purchased from Merck.

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Procedure

The test parameters for the formate endpoint assay adapted to a Cobas Mira S analyzer are outlined in Table I. Sample, standard, or blank (6 µL) is mixed with 64 µL water and 410 µL NAD-reagent. After 5 min incubation (12 cycles) at 37°C, 15 µL of FDH-solution is added. Absorbance is measured at 340 nm every 25 s. The absorbance change (ΔA) attributable to the NADH production when formate is oxidized by FDH is directly proportional to the amount of formate. The blank-corrected absorbance change is multiplied with the theoretical factor based on Beer's law to obtain the formate concentration:

$$c(mg/L) = \Delta A \times factor \qquad \text{Eq. 1}$$

$$factor = \frac{v \times MW}{\varepsilon \times d \times v} = \frac{540\ \mu L \times 46.03\ g.mol^{-1}}{6.3\ L.mmol^{-1}.cm^{-1} \times 0.6\ cm \times 6\ \mu L} = 1096(mg/L) \qquad \text{Eq. 2}$$

[c = formate concentration; V = final volume; MW = molecular weight of formate; ε = extinction coefficient of NADH at 340 nm; d = light path; v = sample volume]

Results

Absorbance change

Figure 1 shows the time course of the reaction. The last absorbance reading was taken 15 min after addition of the enzyme. A plateau was reached for aqueous standard solutions as well as for plasma samples.

Linearity

Aqueous formate standard (10 g/L) was added to normal human plasma to obtain concentrations ranging from 10 to 1000 mg/L with less than 5% volumetric alteration of the matrix. A linear response was obtained for concentrations up to 1000 mg/L covering both nontoxic and highly toxic values. Results are presented graphically in Figure 2. The slope of the regression equation obtained by least-squares linear regression (0.000871 absorbance units.L.mg^{-1}) was very close to the theoretical value of 0.000912 absorbance units.L.mg^{-1} (Eq. 1 and 2), indicating completeness of the reaction under the given conditions.

Imprecision

A within-run replication experiment was performed by analyzing 10 aliquots from two plasma pools spiked with formate (20 and 200 mg/L). Both pools were stored at +4°C and analyzed 12 times over 3 weeks (between-day replication). Within-run and between-day coefficients of variation (CVs) were below 5% at both levels tested (Table II).

Limits of detection (LOD) and quantitation (LOQ)

The LOD and the LOQ were defined as the concentrations corresponding to the mean absorbance change (n = 10) of a blank

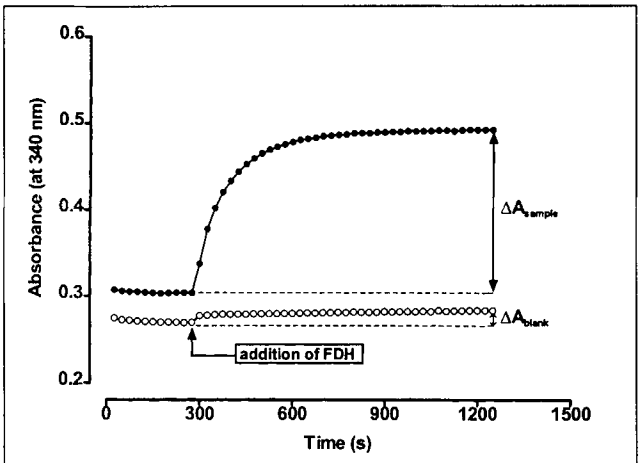

Figure 1. Absorbance change with time for a water blank (— ○ —) and a plasma sample loaded with 200 mg/L formate (— ● —).

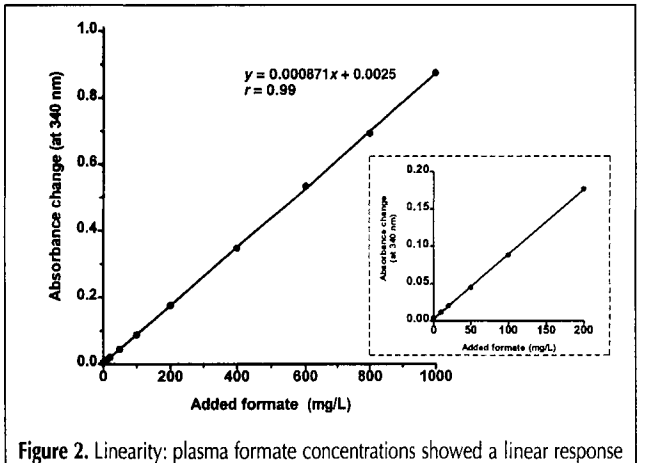

Figure 2. Linearity: plasma formate concentrations showed a linear response with a measurement range up to 1000 mg/L (inset: linearity from 0 to 200 mg/L).

Table I. Cobas Mira S Test Parameters for the Assay of Plasma Formate

General		Analysis	Calculation	Calibration
Measurement mode: absorb	Sample	cycle: 1	Reaction direction: increase	Factor: 1096
Reaction mode: R-S-SR1		volume: 6.0 µL	Calculation step A: endpoint	
Calibration mode: factor		dilution name: H_2O	Readings	first: 11
Wavelength: 340 nm		volume: 64.0 µL		last: 50
Unit: mg/L	Reagent	cycle: 1		
		volume: 410 µL		
	Start R1	cycle: 12		
		volume: 15.0 µL		
		dilution name: H_2O		
		volume: 45.0 µL		

plasma sample plus 3 and 10 standard deviations (SD), respectively. The sensitivity was judged satisfactory with LOD and LOQ values of 7 mg/L and 12 mg/L, respectively.

Recovery

Recovery experiments were carried out by adding formate (200 and 500 mg/L) to blank plasma pools ($n = 4$) or to patient samples containing formate ($n = 4$). Recovery (R) was calculated according to the following formula:

$$R(\%) = \frac{\Delta A_{sample+standard} - \Delta A_{sample}}{\Delta A_{standard}} \times 100(\%) \qquad \text{Eq. 3}$$

Recovery averaged 103.4% (range 98.0%–110.2%), indicating adequate accuracy of the measurements.

Interferences

No significant response was obtained with hemolytic samples (up to 1000 mg/dL hemoglobin added as hemolysate), icteric samples (up to 18 mg/dL bilirubin), and lipemic samples (up to 1000 mg/dL triglycerides) without added formate. When loaded with 200 mg/L formate ($n = 9$), the recovery remained excellent (R = 101.4%; range 98.5%–104.8%).

Methanol (4.0 g/L), ethanol (4.0 g/L), ethylene glycol (2.4 g/L), acetone (1 g/L), and formaldehyde (1 g/L) added to blank plasma samples or the presence of a high lactate level (11 mEq/L) did not interfere before and after addition of 200 mg/L formate, proving good specificity.

Stability of samples and reagents

Plasma samples (200 mg/L formate) stored at ambient temperature for 48 h showed a difference in formate concentration of less than 4% when compared to freshly collected plasma. Patient samples stored at –20°C were stable up to at least 6 months.

Table II. Imprecision Results Obtained Using Spiked Plasma Samples

	Within-run ($n = 10$)		Between-day ($n = 12$)	
Added formate (mg/L)	Mean ± SD (mg/L)	CV (%)	Mean ± SD (mg/L)	CV (%)
20	23.1 ± 1.0	4.4	24.4 ± 1.0	4.1
200	191.5 ± 1.2	0.6	197.4 ± 1.9	1.9

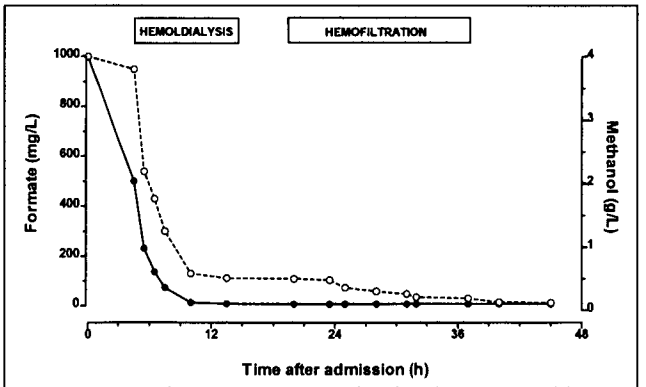

Figure 3. Patient plasma concentrations of methanol (— ○ —) and formate (— ● —) during hospitalization.

Results obtained with NAD- and FDH-reagents stored at +4°C for 2 weeks or with solutions stored at –20°C for at least 3 months were not significantly different from those obtained with freshly prepared reagents.

By adapting the formate assay to the Cobas Mira S analyzer (with low volume for reagent as well as for sample), it was possible to perform 120 tests instead of 20 with the manual procedure. Because of the sustained stability of the frozen reagents, the automated assay is well suited to cope with irregular requests using one aliquot of NAD- and FDH-solution at a time whenever a case of methanol poisoning is presented. Storage of the reagent aliquots at –20°C allows the longer use of one assay kit.

Application

The utility of plasma formate measurement after methanol intoxication is illustrated with the following case.

A 41-year-old man was admitted to a psychiatric unit of a local hospital for ethanol withdrawal. The day after admission he was found comatose. Blood analysis revealed a metabolic acidosis and a high methanol level of 4.0 g/L. After ethanol and sodium bicarbonate treatment were started, the patient was transferred to a university hospital where hemodialysis was performed and folic acid was administered.

Plasma methanol concentrations were determined by gas chromatography–flame-ionization detection during hospitalization of the patient. Formate concentrations were measured after 6 months of storage at –20°C. The formate concentration was 997 mg/L at admission and decreased to endogenous levels (< 12 mg/L) within 10 h of hemodialysis. At that time, the methanol concentration was 0.51 g/L. During subsequent hemofiltration, blood methanol concentration further decreased to 0.12 g/L 45 h after admission (Figure 3). Mild visual disturbances of colored vision characterized the patient's outcome.

In this case, the formate concentration above 500 mg/L at admission and the disturbed ophthalmological examination at discharge confirmed the predictive value of formate measurements in the outcome of methanol intoxication (1).

Conclusions

The Cobas Mira S endpoint enzymatic assay for plasma formate is sensitive (LOD and LOQ of 7 mg/L and 12 mg/L, respectively), reproducible (CVs less than 5%), specific (no interferences) and accurate (mean recovery = 104%). The described method has a broad measurement range of 12 to 1000 mg/L, covering the decision levels of 200 and 500 mg/L indicative of metabolic acidosis and permanent toxicity sequelae, respectively. Because of the stability of the fractionated reagents at –20°C, the method is suitable for semi-urgent, irregular requests and valuable for a more complete assessment of patients poisoned with methanol.

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