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An enzymatic assay with formate oxidase for point-of-care diagnosis of methanol poisoning

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Abstract

Gas chromatographic analysis for quantification of plasma methanol requires laboratory equipment and personnel, and it is typically unavailable in short time notice, especially in low- and middle-income countries. Detection of formate with the enzyme formate oxidase (FOX) is a promising method that can make the diagnosis of methanol poisoning simple and fast. The aims of this study were to test the sensitivity and specificity of a modified FOX-enzyme and to test the specificity of a point-of-care (POC)-model containing FOX-enzyme with samples from patients with metabolic acidosis. The sensitivity and specificity of FOX-enzyme in aqueous solution were evaluated with a spectrometer and by visual detection for colour change. Formate concentrations between 1 and 20 mmol/L were used to test sensitivity, and 18 potentially interfering substances were tested for specificity. The sensitivity of the FOX-enzyme was 100% and the specificity 97%. When specificity of the POC-model was tested, no false positives were detected. As such, the sensitivity and specificity of this modified FOX-enzyme for detection of formate were high. The results with this enzyme confirm the potential for its use in formate analysis as a fast diagnosis of methanol poisoning.

KEYWORDS
bedside testing, diagnosis, formate, methanol poisoning, point-of-care-testing

1 | INTRODUCTION AND BACKGROUND

Methanol poisoning has a high mortality and morbidity, but effective treatment exists provided early diagnosis and initiation of treatment.[1–6] The gold standard for diagnosis is detection of plasma methanol by gas chromatography (GC).[7] This is rarely available around the clock even in large university hospitals in high-income countries, and most often it is not available at all in low- and middle-

income countries. Increased anion gap and osmolal gap are used as surrogate markers instead, but they are unspecific.[8,9] Further, osmolality analyses, done by the proper method with freezing point depression, are also typically not available in low- and middle-income countries.

Methanol itself is not toxic, but it is metabolized by alcohol dehydrogenase via formaldehyde to formic acid, the latter being responsible for the toxic effects. Patients who present late to hospital may have already metabolized most of the methanol to formic acid, and methanol

may therefore be below the detection limit with corresponding high levels of formate.[10,11] The use of an enzymatic method for measuring formate is a good alternative, but currently, it is in limited use as it requires specific enzymes as formate dehydrogenase,[10] a spectrophotometer and capable technicians.[10,12] The typically unspecific clinical features make the diagnosis of methanol poisoning challenging, often with a delayed initiation of treatment as the result. In countries where large outbreaks of methanol poisoning occur regularly, both nonspecific and specific blood tests are often unavailable. Thus, there is an obvious need for a simple and specific blood test for diagnosis of methanol poisoning.

The ideal point-of-care (POC)-test for methanol poisoning should give a diagnosis within minutes from a minimal amount of blood and with a simple visual readout. It should be easy to use, and it should be stable in room temperature. As such, a formate dehydrogenase-based system works in the laboratory, but it has stability issues and would need a cold chain for transport and storage.[13] This is impractical and expensive, thus limiting the potential availability. The enzyme formate oxidase (FOX) can also be used for enzymatic detection of formate, and it is stable at room temperature. However, both the sensitivity and specificity of this enzyme in the diagnosis of methanol poisoning are unknown.

We have tested a modified FOX-enzyme in a simplified POC-model. A formate concentration below 1 mmol/L was set as a threshold value for a negative test, to ensure that endogenous levels were not considered positive.[14] The aims of this study were to test the sensitivity and specificity of a modified FOX-enzyme. Secondly, we wanted to test the specificity of the POC-model with patient samples. Further, we wanted to test the clinical applicability of the POC-model by having clinical staff read the result of samples spiked with various concentrations of formate.

2 | MATERIALS AND METHODS

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[15]

The experiments conducted were divided in two parts: (I) testing the sensitivity and specificity of a modified FOX-enzyme and (II) testing specificity and clinical applicability of a POC-model containing the modified FOX-enzyme.

The principle for the enzymatic method for formate detection is a two-step reaction:

1. $HCOOH + O_2 \rightarrow CO_2 + H_2O_2$ (catalysed by the FOX-enzyme)
2. Leuco dye (colourless) $+ H_2O_2 \rightarrow$ Leuco Dye (colour) $+ H_2O$ (catalysed by horseradish peroxidase)

2.1 | Part I: FOX-enzyme

2.1.1 | Sensitivity and specificity testing of the FOX-enzyme

The sensitivity and specificity of FOX-enzyme were tested in aqueous solution by a spectrometer and by visual detection for a colour change to blue. Broadcom Q-mini spectrometer reading at 653 nm wavelength was used, and the ratio between the measurements after 5 min and at the start was calculated. A ratio ≥ 0.8 was defined as a negative sample and a ratio ≤ 0.7 as a positive sample. The FOX-enzyme solution used was a mixture of the modified FOX-enzyme and other substances that will not be disclosed due to intellectual property rights.

For sensitivity, nine cuvettes were separately filled with a known concentration of formate diluted in NaCl 9 mg/ml before being mixed with the FOX-enzyme solution. The following formate concentrations were used: 1, 2, 4, 5, 6, 8, 10, 15 and 20 mmol/L. For control, nine new cuvettes were filled with the FOX-enzyme solution only. To ensure that the FOX-enzyme could separate a negative sample from a positive sample that was close to the chosen threshold value of 1 mmol/L, a separate test was performed for the following formate concentrations: 0, 0.5, 1.5 and 2.0 mmol/L. The experiment was repeated eight times for 0 mmol/L and 10 times for the other three formate concentrations. This gave 29 samples with formate in the concentration range of 1–20 mmol/L for calculation of sensitivity. In addition to the negative formate samples (0 and 0.5 mmol/L, repeated eight and 10 times respectively), the specificity was tested against 18 different substances in concentrations thought to be clinically relevant (Table 1). This gave 36 samples for calculation of specificity. Eighteen cuvettes were filled with a known concentration of the substances to be tested (Table 1) together with the FOX-enzyme solution. For each cuvette, an associated control with only FOX-enzyme solution was made.

2.2 | Part II: POC-model

2.2.1 | POC-model description

The POC-model is a test strip that requires one drop (40 µl) of whole blood, plasma or serum, which is applied to the

TABLE 1 Substances tested for specificity

Substance	Concentration tested (mmol/L)	Explanation
Acetone	52	Could be present in ketoacidosis
Ascorbate	1000	Could potentially interfere with colour reaction
Beta-hydroxybutyrate	20	Major component in ketoacidosis
Diethylene glycol	10	Toxic alcohol
DL-lactate	20	Cause of lactacidosis[a]
Ethanol	66	Antidote
Ethylenediamin-tetraacetic acid (EDTA)	51	Anticoagulant in blood sample tubes
Ethylene glycol	51	Toxic alcohol
Fomepizole	0.5	Antidote
Glycerol	2	Could be present in metabolic acidosis
Glycolate	20	Cause of acidosis in ethylene glycol poisoning
Isopropanol	51	Toxic alcohol
L-pyroglutamate	20	Metabolite in the glutathione cycle, may cause metabolic acidosis after, for example, paracetamol use
Methanol	96	Toxic alcohol
Methylene blue	0.05	Could potentially interfere with colour reaction
Oxalate	0.02	Metabolite of ethylene glycol
Salicylate	10	Possible cause of metabolic acidosis
Urate	4	Could potentially interfere with colour reaction

[a]Most common cause of metabolic acidosis.

front of the test strip (Figure 1). Red blood cells are removed from whole blood by a separation filter, whereas the enzymatic reaction with the FOX-enzyme takes place at a reactive membrane. The presence of formate will generate a visible colour change at the back of the test strip. To evaluate the result, the test strip was compared with a defined colour scale for semiquantitative detection of formate: negative (corresponding to <1 mmol/L), low positive (1–10 mmol/L) and high positive (> 10 mmol/L) (Figure 2).

2.2.2 | Specificity of POC-model with patient samples

The specificity was tested bedside with one drop of whole blood from 14 patients with metabolic acidosis from various origins, where inclusion criteria was set to a base excess <-5 mmol/L (= base deficit >5 mmol/L). The result was evaluated by visual reading after 4 min against the colour scale for semiquantitative detection (negative, low positive or high positive) by two evaluators (one intensive care [ICU] nurse and one author YEL).

2.2.3 | Clinical applicability of POC-model

Four ICU doctors and two ICU nurses were randomly selected to read the results of the POC-model. None of the participants were familiar with the POC-model or how to read the result in advance. Four different blood samples were tested: Two negative samples, one sample spiked with 3 mmol/L formate (low positive) and one spiked with 20 mmol/L formate (high positive). One drop of sample was added to the test strip and repeated for each sample. After 4 min, the participant individually assessed the results of the test by visually defining whether each test was negative, low positive or high positive. This was repeated for each participant, and the true result was blinded to them. Fleiss' kappa was used to evaluate the inter-rater reliability between the participants since there were more than two of them.[17] Kappa values between 0.61 and 0.80 are considered substantial strength of agreement, and more than 0.80 is considered almost perfect strength of agreement.[18] IBM SPSS® Statistics for Windows (Armonk, NY, USA) version 27 was used for the statistical calculations.

2.2.4 | Ethical considerations

Specificity of the POC-model with patient samples was a quality control study and approved by the Data Protection Officer at Oslo University Hospital (case number 21/15801). Experiments involving biological material was destroyed immediately after use.
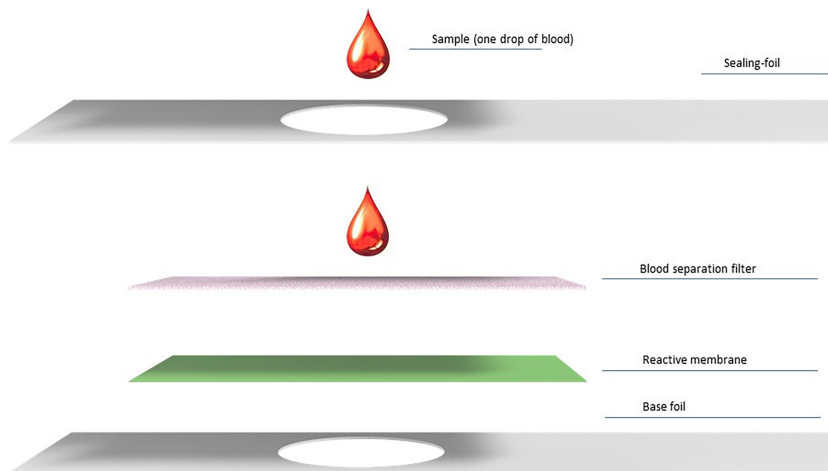
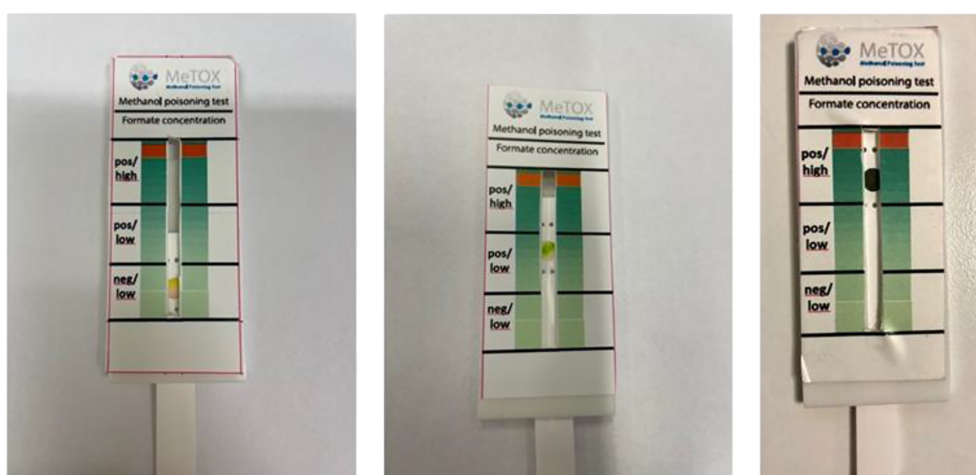FIGURE 1 Illustration of principles for point-of-care model

FIGURE 2 Illustration of point-of-care model with three level scales for semiquantitative detection of formate; negative <1 mmol/L, low positive 1–10 mmol/L and high positive >0 mmol/L. *Note*. Adapted from "Formate test for bedside diagnosis of methanol poisoning," by KE Hovda et al., 2021, *Basic Clin Pharmacol Toxicol*., 129, p. 87[16]

3 | RESULTS

3.1 | Part I: FOX-enzyme

3.1.1 | Sensitivity and specificity testing of the FOX-enzyme

All 29 samples with formate concentrations higher or equal to 1 mmol/L were evaluated as positive with a ratio ≤0.7 after 5 min (Figure 3), giving a sensitivity of 100%. The average ratio was 0.4 (range 0–0.7). The results for samples with low formate concentrations near the threshold value (1 mmol/L) are presented in Figure 4. Of note, the positive samples with formate 1.5 and 2 mmol/L have a ratio ≤0.7 after 5 min and can be separated from the negative samples 0 and 0.5 mmol/L with a ratio ≥0.8.

With regard to the nine formate concentrations tested between 1 and 20 mmol/L, they all gave a visual colour change to blue after 5 min. The results for 2, 5 and 10 mmol/L are presented in Figure 5. Note that samples with formate changed colour to blue indicating a positive sample.

Regarding specificity, only one of the 36 negative samples gave a false positive result, giving a specificity of 97%. The sample with isopropanol had a ratio of 0.5 (positive sample) and changed colour, but it immediately turned green instead of a gradual blue development.

3.2 | Part II: POC-model

3.2.1 | Specificity of POC-model with bedside patient samples

All samples from patients with metabolic acidosis of other origins than methanol poisonings were evaluated as negative: six patients had diabetic ketoacidosis, three had confirmed ethylene glycol poisoning, one had renal

FIGURE 3 Sensitivity of formate oxidase (FOX)-enzyme in aqueous solutions evaluated with a spectrometer for nine formate concentrations between 1 and 20 mmol/L. Y-axis is the ratio between the measured value at a given time and start ($T = 0$). A ratio ≥ 0.8 after 5 min was defined as a negative sample and a ratio ≤ 0.7 as a positive sample.
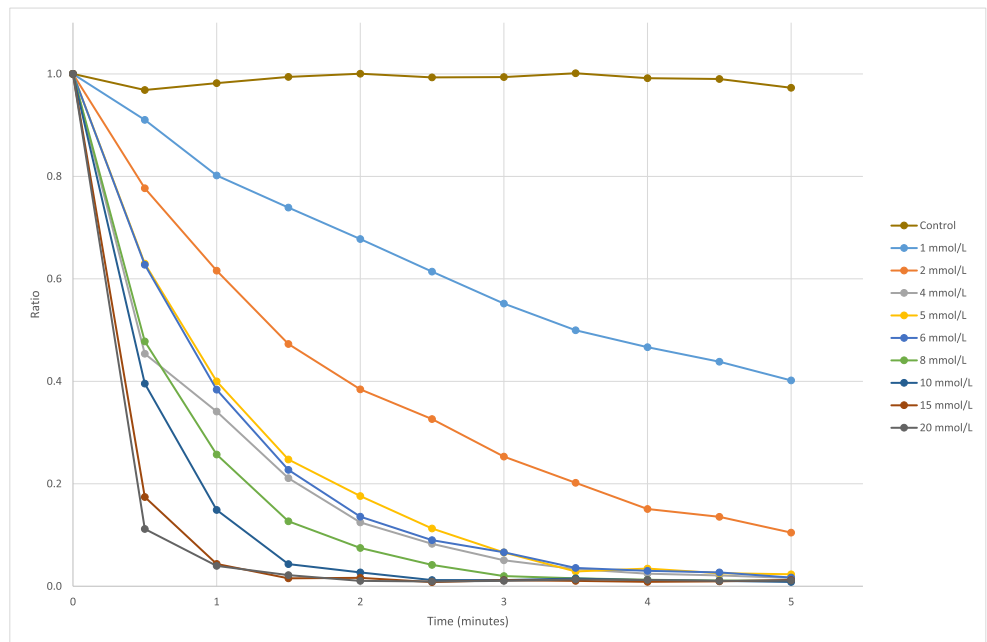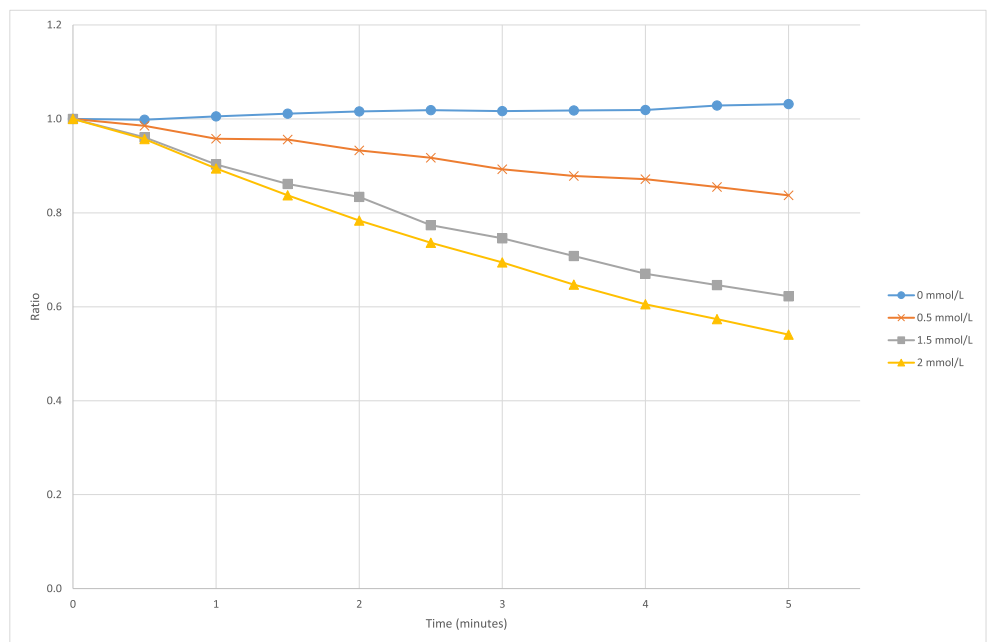

FIGURE 4 Sensitivity of formate oxidase (FOX)-enzyme in aqueous solutions evaluated with a spectrometer for low concentrations of formate. Y-axis is the ratio between the average measured value for each concentration at a given time and start ($T = 0$). A ratio ≥ 0.8 after 5 min was defined as a negative sample and a ratio ≤ 0.7 as a positive sample.

failure with metabolic acidosis, one had lactic acidosis and three had metabolic acidosis of unknown origin without suspicion of methanol poisoning.

3.2.2 | Clinical applicability of the POC-model

The high positive (20 mmol/L), low positive (3 mmol/L) and one negative sample were identified correctly by all six clinical staff participants. The second negative sample was identified correctly by 5/6 clinical staff participants, whereas one assessed it as low positive.

Comparing the inter-rater reliability between the six clinical staff participants gave a Fleiss' kappa value of 0.87 ($p < 0.001$), 95% CI (0.69–1.05), suggesting an almost perfect strength of agreement.[18]

4 | DISCUSSION

The present study evaluated the sensitivity and specificity of this modified FOX-enzyme in aqueous solution. The sensitivity for formate was high in the concentration range of 1–20 mmol/L, being most relevant in clinical practice. During a methanol outbreak in Norway, all
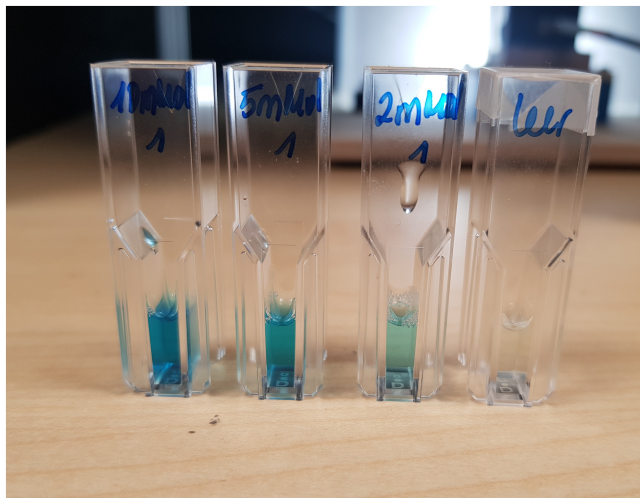
FIGURE 5 Illustration of the reaction between formate and formate oxidase (FOX)-enzyme that gives a colour change to blue after 5 min. From left; formate 10 mmol/L; 5 mmol/L; 2 mmol/L and control (without formate)

symptomatic patients had a formate concentration above 10 mmol/L, whereas four patients without symptoms had a formate level between 0.5 and 8.3 mmol/L.[10] From the large outbreak in the Czech Republic with 101 hospitalized patients, the median formate concentration on admission was 13.4 mmol/L, whereas the highest was 25.2 mmol/L.[11] Formate concentrations were higher in patients with clinical features. Those with visual disturbances had a median value of 15.2 mmol/L, and those with dyspnoea had a median value of 15.4 mmol/L.[12] One patient presented with visual disturbances and a formate concentration of 3.7 mmol/L. This case most likely represented an analytical error, as it did not correspond to the arterial blood gas and anion gap on arrival (personal communication with main author S. Zakharov).

The specificity of the FOX-enzyme in aqueous solution was high with only one false positive. Isopropanol had a ratio of 0.5 after 5 min and changed colour immediately to green when the FOX-enzyme solution was added. This colour was different from the enzymatic reaction that occurred over time in the presence of formate. The fact that the colour change occurred immediately and to a different colour may indicate that this was a different type of reaction. The substances tested for specificity represent relevant differential diagnoses in metabolic acidosis, substances potentially interfering with the colour reaction or common additives to blood sampling tubes (Table 1).

Previous attempts with enzymatic assays for detection of methanol have shown that the specificity was low towards ethanol.[19,20] In a study from Hack et al., they used a commercial product with alcohol oxidase for detection of

ethanol in saliva, to test if methanol, isopropanol and ethylene glycol could be detected when added to plasma.[19] They found that the sensitivity for methanol was good, but it did not distinguish between concomitant ingestion of ethanol. Similarly, when Shin et al. used alcohol oxidase in a liquid-based system to test for methanol and ethanol in saliva, it was not possible to separate between the two alcohols.[20] Such co-ingestion is very common, and avoiding a false positive result is crucial. In the present study, the FOX-enzyme in aqueous solution did not react with ethanol and so did not give a false positive result. When the POC-model was tested bedside with samples from 14 patients with metabolic acidosis of various aetiologies, none gave a false positive result. The POC-model is meant to be a screening tool in metabolic acidosis of unknown origin to verify, or exclude, methanol as the cause. The present data thus support its usefulness as a diagnostic tool.

When testing the clinical applicability of the POC-model, the samples were correctly identified by the clinical staff, except for one participant who identified one negative sample as a low positive. With a low positive test (1–10 mmol/L), the patient is likely asymptomatic.[10,12] This indicates that treatment should not be initiated. However, a control test is then indicated after 2–4 h to evaluate whether the formate concentrations are steady, decreasing/normalizing or increasing. In the latter case, treatment should be initiated. This will ensure that nonsignificant intakes of methanol do not lead to unnecessary treatment, whereas the control after a given period would indicate if the formate concentration is on the rise. If the patient develops clinical features before the control test, an additional test should be run immediately, to find out whether the features are because of methanol poisoning or not. Therefore, this one negative sample that was erroneously read as low positive would in the clinical situation have triggered a following test after 2–4 h and would thus not have led to unnecessary treatment. In case of uncertainty, as to whether the test is negative or low positive, we recommend it to be interpreted as a low positive and thus be followed by a repeated test.

The negative sample identified correctly by all clinical staff was a blood sample from a patient that previously had a false positive methanol result with the gold standard GC-mass spectrometry (GC–MS). The initial false positive methanol result was followed by a second false positive result on GC–MS. This led to initiation of antidote treatment of this severely acidotic patient. Treatment was continued until repeated reanalysis with GC–MS showed a negative result, also later confirmed by a formate assay by GC–MS. The patient in question would clearly have been a candidate for this POC-model due to the metabolic acidosis of unknown origin. As shown in our study, everyone

evaluated this as negative sample, and the patient would not have received the antidote treatment based on this.

In most of countries where large outbreaks of methanol poisoning occur, there is limited or no possibility of confirming or eliminating the diagnosis. Treatment facilities are also often limited both as regards dialysis and ICU capacity. This is also true for the preferred antidote, fomepizole, which is typically not available due to its current cost. As such, the presented POC-model may offer new possibilities for diagnosis and treatment and also a chance to avoid unnecessary treatment. The test is based on detection of the toxic metabolite formate rather than the parent alcohol. Without formate present, methanol poisoning will not give any clinical features.[10,12] The high sensitivity of the FOX-enzyme will detect even low concentrations of formate, and it allows detection even hours before the clinical features become apparent. Further, patients who present late after ingestion may not have any methanol left on admission due to its elimination by metabolism. In a study by Zakharov et al., two of the patients had a methanol concentration below detection limit, but detection of formate confirmed methanol poisoning.[12] On the other hand, a negative formate test does not rule out methanol poisoning. This may be seen in patients who present early or have co-ingested ethanol.[2] However, these patients would have no clinical features from the methanol poisoning. An algorithm for the clinical use of a formate analysis in diagnosing methanol poisoning has earlier been suggested.[7]

The POC-model will also offer opportunities in countries that have facilities to confirm the diagnosis. In the absence of a 24-h service, or if the samples must be sent to another hospital for analysis, this test enables a faster diagnosis. Even in large university hospitals where specific analyses are available around the clock, the result using the POC-model may be obtained bedside within less than 5 min rather than hours. We recently published the first case report where this POC-model was used in clinical practice on a methanol poisoned patient[16]: The test showed high positive formate even before the result of the blood gas analysis returned, and antidote treatment was started immediately.

Fomepizole is the preferred antidote for treatment of methanol poisoning, but it is not available in most low- and middle-income countries due to the current price. Ethanol is therefore often used as the only available antidote.[21] The main disadvantages of using ethanol are the difficult dosing and the need for monitoring of plasma concentration to ensure the recommended ethanol level of 22 mmol/L (100 mg/dl).[5,6] In the absence of ethanol analyses, the current POC-model for formate detection may also be used to monitor the antidote effect. Increased

formate concentrations indicate that the metabolism of methanol is not blocked, and the ethanol dosage needs to be increased.

In our study, we have shown that this modified FOX-enzyme has a high sensitivity and specificity, thus being promising for use in diagnosing methanol poisoning. Further development and clinical trials are warranted.

5 | LIMITATIONS

The sensitivity and specificity of the FOX-enzyme is only tested in aqueous solutions and not whole blood, plasma, or serum. Further, when specificity was tested with the 18 mentioned substances, only one defined concentration per substance—although high—was tested. It is therefore unknown whether more extreme concentrations of the substances in question can have an effect. When specificity of the POC-model was tested bedside with samples from patients with metabolic acidosis, we only included 14 patients. In addition, none of them had alcoholic ketoacidosis, which is a very important differential diagnosis. However, neither lactate nor beta-hydroxybutyrate or ethanol gave a false positive result when FOX-enzyme specificity was tested separately. The clinical applicability of the POC-model was only tested with six ICU personnel and is hence not to be considered as a full usability test, rather as a pilot study to show the feasibility of such a POC-test.

6 | CONCLUSION

The present data showed a high sensitivity and specificity of a modified FOX-enzyme in aqueous solutions for detecting formate. When a POC-model containing FOX-enzyme was tested for specificity with bedside samples from patients with metabolic acidosis of other origin than methanol poisoning, no false positives were detected. The current method may provide opportunities for quick and easy diagnosis of methanol poisoning where this is not possible today, and it can reduce the time from sampling to obtaining the result, even where this is available around the clock.

DATA AVAILABILITY STATEMENT

Data from the current study will be available upon request.

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