# Determination of absolute hydrogen peroxide concentration by spectrophotometric method

There is considerable current interest in oxidative stress applicable to humans. One important reactive oxygen species generated in the body is $H_2O_2$. It was reported that substantial amount of $H_2O_2$ was excreted in urine[1–3], even in neonates[4]. Therefore, it was hypothesized earlier that measurement of $H_2O_2$, especially in urine, could be a valuable biomarker for the generation of $H_2O_2$ and the degree of oxidative stress *in vivo*[5,6].

The FOX-2 method has been used earlier to detect $H_2O_2$ in urine[3,7]. The basic principle of this method is oxidation of ferrous ions by the sample oxidizing agents to ferric ions, which bind with xylenol orange to give a coloured complex[8,9]. Gupta *et al.*[10] had developed the basic method in 1975 which was later on elaborated by Wolf and coworkers[11]. Though the method has been used to detect $H_2O_2$, hydroperoxides can also oxidize ferrous ions to ferric ions. Therefore, this method has been used successfully to measure plasma hydroperoxide concentration[9,10,12]. $H_2O_2$ is possibly absent in normal plasma due to the presence of its degradative systems like catalase[7,13].

In the FOX-2 method 90 µl of sample, 10 µl of methanol and 900 µl of xylenol orange (Sigma Chemical Co.) reagent[14] containing ferrous ions were added successively and absorbance was noted at 560 nm (ref. 3). Therefore, the classical FOX-2 method measured any substance that was converting $Fe^{2+}$ to $Fe^{3+}$, irrespective of their chemical nature. Ten µl of methanol was replaced by 10 µl of 10 mM triphenylphosphine earlier to reduce plasma hydroperoxides, and this modification of FOX-2 was used to measure plasma hydroperoxide concentration more accurately[9]. In this correspondence we have modified the method by performing the assay in the presence or absence of 10 µl of catalase. This was done by replacement of 10 µl of methanol with 10 µl of catalase solution to measure absolute $H_2O_2$ concentration in biological samples. Catalase reagent was prepared by dissolving 1 mg of catalase powder (22,000 U/mg from Sigma) in 10 ml of 25 mM phosphate buffer pH 7.0, containing 2 mg/ml bovine serum albumin and stored in aliquots at –20°C.

Catalase is an antioxidant enzyme catalysing the following reactions[15].

Reaction A – $2H_2O_2 \rightarrow 2H_2O + O_2$,

Reaction B – $ROOH + AH_2 \rightarrow ROH + A + H_2O$.

($AH_2$ is a hydrogen donor like methanol.)

For both the reactions catalase–$H_2O_2$ complex was formed first[15]. Reaction A proceeded exceedingly rapidly with a rate constant $k \approx 10^7$/mol/s (refs 15 and 16), whereas the rate of reaction B was quite slow, with $k \approx 10^2$–$10^3$/mol/s (refs 15 and 16). Therefore, the second reaction is insignificant when compared to the first. Moreover, *in vitro* many of the hydrogen donors act as inhibitors of catalase[15]. Hence replacement of 10 µl of methanol with 10 µl of catalase solution definitely makes the FOX-2 assay more specific for $H_2O_2$ measurement. The absolute $H_2O_2$ concentration is calculated from the absorbance difference of FOX-2 and modified FOX-2. The standard plot of $H_2O_2$ was prepared fresh each time and concentration of $H_2O_2$ stock solution was checked using molar extinction coefficient of $H_2O_2$ at 240 nm as 43.6 $M^{-1}$ $cm^{-1}$ (ref. 14).

Urine samples were collected from healthy human volunteers. Cerebrospinal fluid (CSF) and ascitic fluid were taken from Clinical Biochemistry Division, Kasturba Medical College, Manipal. Samples were examined within 30 min of collection. Morning fasting samples were taken from normal subjects. Smokers were excluded from the study. All reagents were of analytical grade. All glasswares were cleaned with warm concentrated nitric acid[17] and deionized water before an experiment.

Recovery experiments were done with urine, CSF and ascitic fluid to check whether there was an increase in absorbance with increasing concentration of $H_2O_2$ (Figure 1). Biological fluids can contain substances that can degrade $H_2O_2$. If such $H_2O_2$ degradative systems like catalase, glutathione peroxidase or free haeme exist, then residual $H_2O_2$ concentration in any biological fluid is far from reality. Recovery experiments were designed to understand the presence of $H_2O_2$ degradative systems in biological fluids like urine, CSF and ascitic fluid. These experiments with normal human urine, CSF and ascitic fluid showed linear and gradual increase in absorbance as $H_2O_2$ concentration was increased, signifying the absence of $H_2O_2$ degradative systems and a baseline $H_2O_2$ concentration in these biological fluids. We have studied the recovery percentage of $H_2O_2$ in normal human urine which is 100%, signifying the complete absence of $H_2O_2$ degradative systems in this biological fluid.

It was reported earlier that urine of normal human subjects contains measurable amount of $H_2O_2$ (ref. 7). This experiment was a repetition of these studies with and without modification. $H_2O_2$ concentration in urine of normal human volunteers was determined by FOX-2 and modified FOX-2 method (Table 1). The results showed that normal human urine contains substantial amounts of $H_2O_2$. The values with the modified FOX-2 method were lower than the original FOX-2 method.

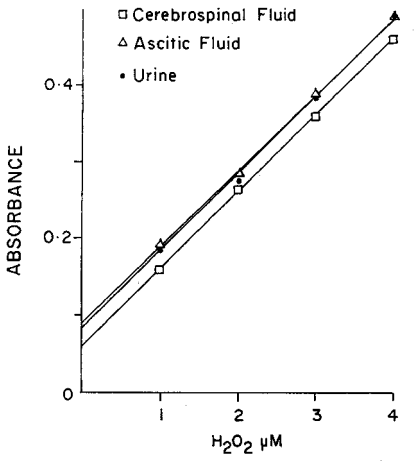


**Figure 1.** Recovery experiments with urine, CSF and ascitic fluid. Increasing concentrations of $H_2O_2$ were added to 90 µl of pooled urine (•), CSF (□) or ascitic fluid (Δ). Ten µl of methanol was added to it immediately, mixed well and then 900 µl of FOX-2 reagent was added. Each tube was incubated for 30 min, centrifuged at 5000 rpm for 10 min and absorbance was measured at 560 nm.

**Table 1.** Estimation of $H_2O_2$ concentration in urine of normal human volunteers by FOX-2 and modified FOX-2 methods. FOX-2 assay was performed as in Figure 1. Concentrations of $H_2O_2$ obtained from standard plots are given in column (a). Modified FOX-2 assay was done by replacing 10 µl of methanol with 10 µl of catalase (22 units). The absorbance value obtained was subtracted from the absorbance value of the classical FOX-2 method. The value obtained was used to calculate the concentration of $H_2O_2$ from standard plots and is given in column (b)

| | $H_2O_2$ concentration (µM) by | |
|---|---|---|
| Age (years) | FOX-2 method (a) | Modified FOX-2 method (b) |
| 20 | 111.1 | 71.1 |
| 23 | 104.4 | 81.1 |
| 21 | 137.8 | 86.2 |
| 23 | 53.3 | 26.7 |
| 22 | 80.0 | 60.7 |
| 19 | 75.5 | 59.6 |
| 26 | 101.1 | 82.9 |
| 31 | 113.3 | 70.0 |
| 25 | 83.3 | 83.3 |
| 23 | 80.0 | 75.0 |
| 21 | 126.7 | 86.6 |
| 24 | 38.9 | 38.8 |
| 20 | 74.4 | 52.0 |
| 26 | 94.4 | 72.0 |
| 22 | 77.8 | 68.2 |

Previously, FOX-2 method was used to detect urinary $H_2O_2$ (ref. 3). The concentration of $H_2O_2$ detected by FOX-2 method was compared with that by the oxygen-electrode method. The oxygen-electrode method gave significantly lesser value than FOX-2 method[3]. Here, $H_2O_2$ detected by modified FOX-2 method was always less than that detected by the original version of FOX-2 method. The oxygen-electrode measures $H_2O_2$ depending upon the catalytic activity of catalase[3]. Modified FOX-2 also measures $H_2O_2$ depending upon the catalytic activity of catalase. Therefore, catalase modification of FOX-2 enables us to get an absolute $H_2O_2$ concentration in biological samples.

The recovery experiments with CSF, ascitic fluid and urine gave linear plots (Figure 1). Hence $H_2O_2$ degradative system was not present in these biological samples and its measurement was possible in these biological fluids by this method. Among these biological fluids, we have determined $H_2O_2$ concentration in normal human urine.

In earlier reports where urinary $H_2O_2$ was reported in normal subjects, a value as high as 100 µM was noted, and most of the samples contained more than 10 µM of $H_2O_2$ (refs 3 and 7). But by modification of the FOX-2 method, in none of our samples from normal human volunteers was there more than 100 µM $H_2O_2$. Using the original version of FOX-2 method, six samples showed a value of more than 100 µM. Therefore, we infer that the value obtained by the modified version was the correct value of $H_2O_2$ concentration because of replacement of methanol by catalase. Moreover, in the previous studies spot-random samples of urine were collected without taking into consideration any dietary history[3,7]. It is possible that like many other biochemical parameters, $H_2O_2$ excretion varies according to circadian rhythm. And, it is proved that diet affects $H_2O_2$ concentration in biological samples[7,18]. Hence we have estimated $H_2O_2$ concentration in normal human urine collected in the morning in fasting state. This mode of collection of samples may have affected our decreased value in the modified version of FOX-2. Any oxidative stress can alter $H_2O_2$ concentration; therefore, we have estimated $H_2O_2$ concentration in urine collected from normal individuals, excluding smokers as smoking is a proved case of oxidative stress[19,20]. So we conclude that in basal conditions, urinary $H_2O_2$ is substantially high, but may not be as high as 100 µM.


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Received 20 August 2002; revised accepted 30 September 2002


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