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A Headspace Gas Chromatographic Method for the  
Determination of Total Ketone Body Concentration in Blood:  
Analysis of Samples Collected from Victims of Sexual Assault

by

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# A HEADSPACE GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF TOTAL KETONE BODY CONCENTRATION IN BLOOD: ANALYSIS OF SAMPLES COLLECTED FROM VICTIMS OF SEXUAL ASSAULT<sup>1</sup>

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## ABSTRACT

Modification of the headspace gas chromatography method used for ethanol measurement allowed accurate quantitation of acetone in blood. Enzymatic conversion of 3-hydroxybutyrate to acetoacetate and subsequent conversion of the acetoacetate to acetone provided a specific, reproducible method for total ketone body determination. Analysis of blood obtained from sexual assault victims showed that the mean total ketone body concentration ( $310.1 \mu\text{mol/L}$ ) for all cases was higher than the mean total for blood from control individuals ( $213.3 \mu\text{mol/L}$ ). There was no significant difference in the total ketone body concentration between the sexual assault victims with visible signs of injury and those with no visible signs of injury ( $p > 0.5$ ). Consumption of ethanol by both groups of sexual assault victims inhibited production of all constituents of the ketone body pool.

## RÉSUMÉ

Une modification de la méthode de dosage de l'éthanol par chromatographie gazeuse, nous permet l'analyse quantitative précise de l'acétone dans le sang. La conversion enzymatique du 3-hydroxybutyrate en acétoacétate et la transformation subséquente de l'acétoacétate en acétone constituent une méthode spécifique et reproductible pour la détermination des corps cétoniques. L'analyse du sang de victimes d'agression sexuelle a démontré que la concentration moyenne des corps cétoniques ( $310.1 \mu\text{mole/L}$ ) dans tous les cas, était supérieure à la concentration sanguine moyenne des individus contrôles ( $213.3 \mu\text{mole/L}$ ). Aucune différence significative n'a pu être démontrée entre les concentrations des corps cétoniques chez les victimes d'agression sexuelle avec blessures apparentes et chez celles ne démontrant aucune blessure apparente ( $p > 0.5$ ). L'absorption d'éthanol par les deux groupes de victimes d'agression sexuelle a inhibé la production de tous les constituants de l'ensemble des corps cétoniques.

## INTRODUCTION

Ketone body production, in man, is initiated in the liver through the partial oxidation of fatty acids to acetyl-CoA (1,2). Acetyl-CoA is converted into free acetoacetate and 3-hydroxybutyrate, which are transported by the blood to the peripheral tissues where they can be oxidized via the tricarboxylic acid cycle (3). Acetone, acetoacetate and 3-hydroxybutyrate, make up the total ketone body pool. Clinically, disturbances in energy metabolism, either intentionally (eg. fasting) or through a disease process (eg. diabetes), can affect the quantity and distribution of the individual components of the ketone body pool (4, 5). Elevated ketone body concentrations have been reported after intravenous infusions of epinephrine or norepinephrine (6-8) and as a result of physical injury (9). Recent observations of an increased frequency of detectable acetone in the blood and urine of victims of sexual assault (10) and in the blood of victims of hypothermia deaths (11) have

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suggested that the analysis of ketone bodies may aid in the forensic interpretation of events related to these incidents.

Determination of acetone concentrations alone has traditionally been of limited clinical usefulness (12, 13) and does not accurately reflect trauma induced changes in metabolism in the presence of ethanol (10). Analysis of all components of the ketone body pool is necessary to provide a comprehensive picture of fatty acid metabolism.

Headspace gas chromatography (HSGC) has been used by some investigators for the detection of the three constituent members of the ketone body pool (14, 15, 16). Although HSGC is an accurate and sensitive method for the quantitation of acetone, conversion of 3-hydroxybutyrate and acetoacetate to acetone has involved either acid hydrolysis, which can lead to the decomposition of 3-hydroxybutyrate to products other than acetone (15) and the formation of undesirable byproducts (15, 17), or the use of proprietary enzyme systems (16, 17). Here we describe a hybrid method of total ketone body determination involving an adapted enzymatic method (16) for the conversion of 3-hydroxybutyrate to acetoacetate and the subsequent determination of acetoacetate after conversion to acetone by heating (14). Preliminary results on total ketone body determination of victims of sexual assault are also reported.

## MATERIALS AND METHODS

### Apparatus

A Perkin-Elmer Multifract F-45 headspace gas chromatograph equipped with a flame ionization detector was used to determine the ethanol concentrations. The column was a 1.8 m × 3.0 mm (ID) stainless steel column packed with Carbopac C 60/80 mesh coated with 0.2% Carbowax 1500. The carrier gas (nitrogen) and hydrogen pressures were 1.2 bar. The operating temperatures were; oven: 90°C, injector port: 150°C, dosing system: 170°C, and automatic turntable: 45°C.

A Perkin-Elmer Multifract F-40 headspace gas chromatograph equipped with a flame ionization detector was used to determine the acetone concentrations. The column was a 1.8 m × 3.0 mm (ID) stainless steel column packed with Carbopac C 60/80 mesh coated with 0.2% Carbowax 1500. The carrier gas (nitrogen) pressure was 1.0 bar and the hydrogen pressure was 1.5 bar. The operating temperatures were; oven: 80°C, injector port: 150°C, dosing system: 170°C, and automatic turntable: 45°C.

### Reagents

Lithium acetoacetate, L-(+)-lactic acid, DL-3-hydroxybutyrate, and pyruvate (Type I-S) were from Sigma Chemical Co., St. Louis, MO, 63178. Acetone and other chemicals were analytical reagent grade products from Caledon Labs Georgetown, Ontario.

The 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30; grade II; from *Rhodopseudomonas spheroides*; 5.3 U/mg protein), L-lactic dehydrogenase (EC 1.1.1.27; type XI; from rabbit muscle; 830 U/mg protein) and B-NAD<sup>+</sup> (grade III) were from Sigma Chemical Co., St. Louis, MO.

Phosphate solutions were kept at room temperature. Acetoacetate and lactate were stored at -20°C. All other reagents were refrigerated at 4°C.

The enzyme reagent was prepared as outlined by Kimura et al. (16). The reagent consisted of 16.05 mL of 3-hydroxybutyrate dehydrogenase, 1.33 mL of lactic dehydrogenase, 1.9 mL

of B-NAD<sup>+</sup>, 3.8 mL pyruvate (80 mmol/L, pH 7.0) and 15.2 mL of Na<sub>3</sub>PO<sub>4</sub> (0.4 mol/L, pH 13.7). The reagent was stored at 4°C until use.

### Sample Collection

Control blood samples (n=8) were collected from living individuals and were negative for ethanol. Blood samples from the sexual assault victims were obtained from the uniform kit issued to all police agencies in Ontario for the collection of evidence from the victims of sexual assault. The blood was normally collected in a grey top Vacutainer® (XF 947) containing 100 mg sodium fluoride and 20 mg potassium oxalate. Average collection time for the sexual assault victims was approximately 8.6±7.1 (mean±SD) hours after the assault.

### Methods

To measure the concentration of acetone in the blood or aqueous solutions, 0.5 mL of sample and 0.1 mL of a tertiary butanol internal standard were sealed in a vial. Samples were allowed to equilibrate in the automatic turntable for at least thirty minutes prior to analysis. All samples were analyzed in duplicate and the mean result was reported in each case. The coefficient of variation for the aqueous control standards of ethanol and acetone, analyzed under the described conditions, was approximately 1.5%.

Conversion of acetoacetate to acetone was accomplished by incubating the vial containing the sample and the internal standard in an oven at 90°C for predetermined periods of time. The sample was cooled and allowed to equilibrate in the automatic turntable for at least thirty minutes prior to analysis.

To determine the concentration of 3-hydroxybutyrate the sample, internal standard, and 0.1 mL of the enzyme reagent were incubated in a sealed vial for 30 minutes at 37°C. This reaction results in an equimolar conversion of 3-hydroxybutyrate to acetoacetate. The acetoacetate concentration was then determined as outlined above.

## RESULTS

### Determination of Acetone Concentration

Aqueous standards were prepared by spiking with acetone at concentrations ranging from 20 μmol/L to 500 μmol/L. The recovery positively correlated with the expected values ( $r^2=0.9999$ ) throughout the entire range (Figure 1) and approximated 100% through all concentrations (Table 1A). Analysis of two spiked control bloods, (A and B), corrected for endogenous acetone, showed a similar correlation over the range tested (Figure 1). At concentrations below 100 μmol/L there were fluctuations in the total acetone recovered (58–181%) while at concentrations greater than 100 μmol/L recovery was consistent and approached 95% of the level of the aqueous standards (Table 1A).

### Determination of Acetoacetate Concentration

Free acetoacetate formed in vivo by the oxidation of fatty acids is subsequently reduced enzymatically to 3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase or converted to an active form, acetoacyl CoA, for further processing in the tricarboxylic acid cycle. In refrigerated, preserved blood samples enzymatic reduction does not occur and spontaneous decarboxylation of acetoacetate to acetone proceeds at a negligible rate (17). Van Stekelenberg and De Bruyn (14) were able to accelerate the rate of acetoacetate decarboxylation by heating serum samples to 90°C for 1 hour. Aqueous samples, spiked with acetoacetate

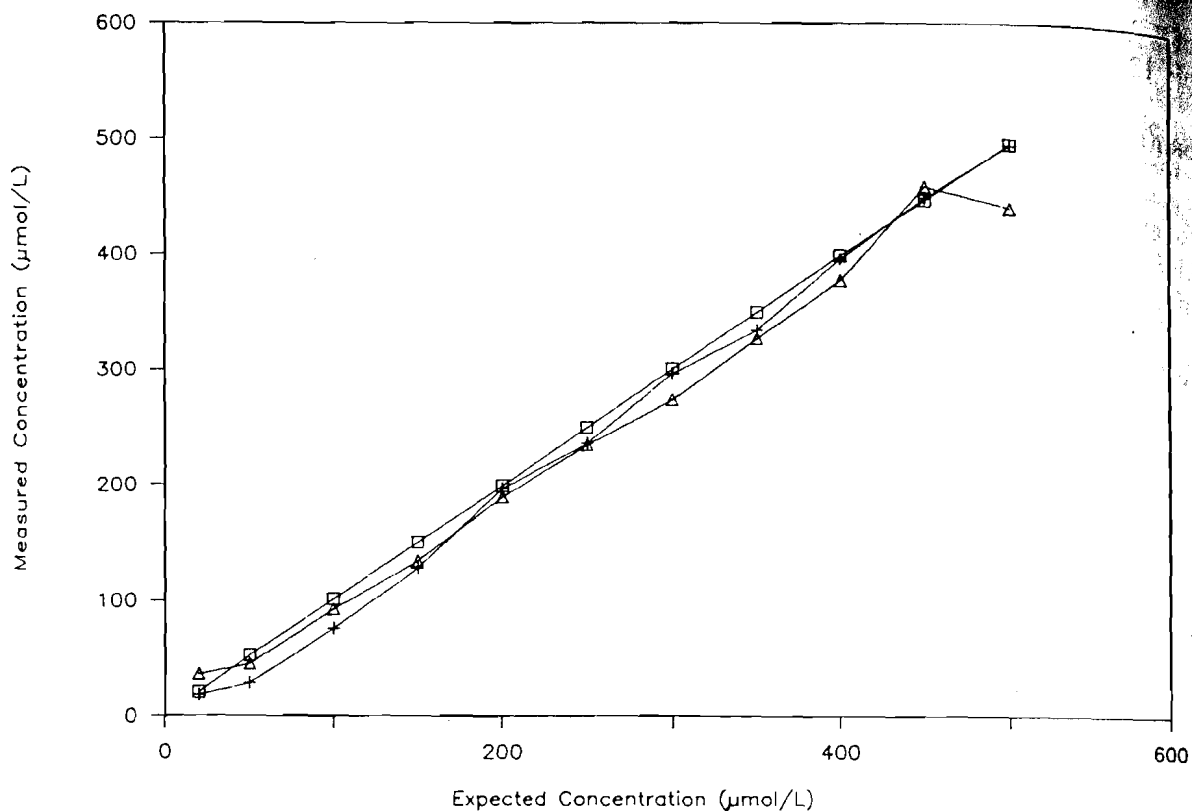


Figure 1. Acetone recovery from spiked aqueous solution (□----□), spiked blood A (△----△), and spiked blood B (+----+) as determined by headspace gas chromatography.

TABLE 1

ANALYTICAL RECOVERY AND PRECISION OF THE HEADSPACE GAS CHROMATOGRAPHIC METHOD

Ketone	Sample	Mean Recovery, %	CV, %
A	Aqueous	101.25	2.54
	Blood A	93.40	3.95
	Blood B	94.14	7.98
B	Aqueous	84.17	1.92
	Blood A	71.36	6.31
	Blood B	76.71	3.67

at a concentration of 500  $\mu\text{mol/L}$ , were heated in an oven to temperatures ranging from 45°C to 90°C. At varying times samples were removed, analyzed on the gas chromatograph and the acetone concentration determined.

Heating samples to temperatures less than 65°C did not appreciably increase the rate of conversion of acetoacetate to acetone (Figure 2). A temperature of 90°C was necessary to facilitate an 83% conversion to acetone within 250 minutes. Further heating did not

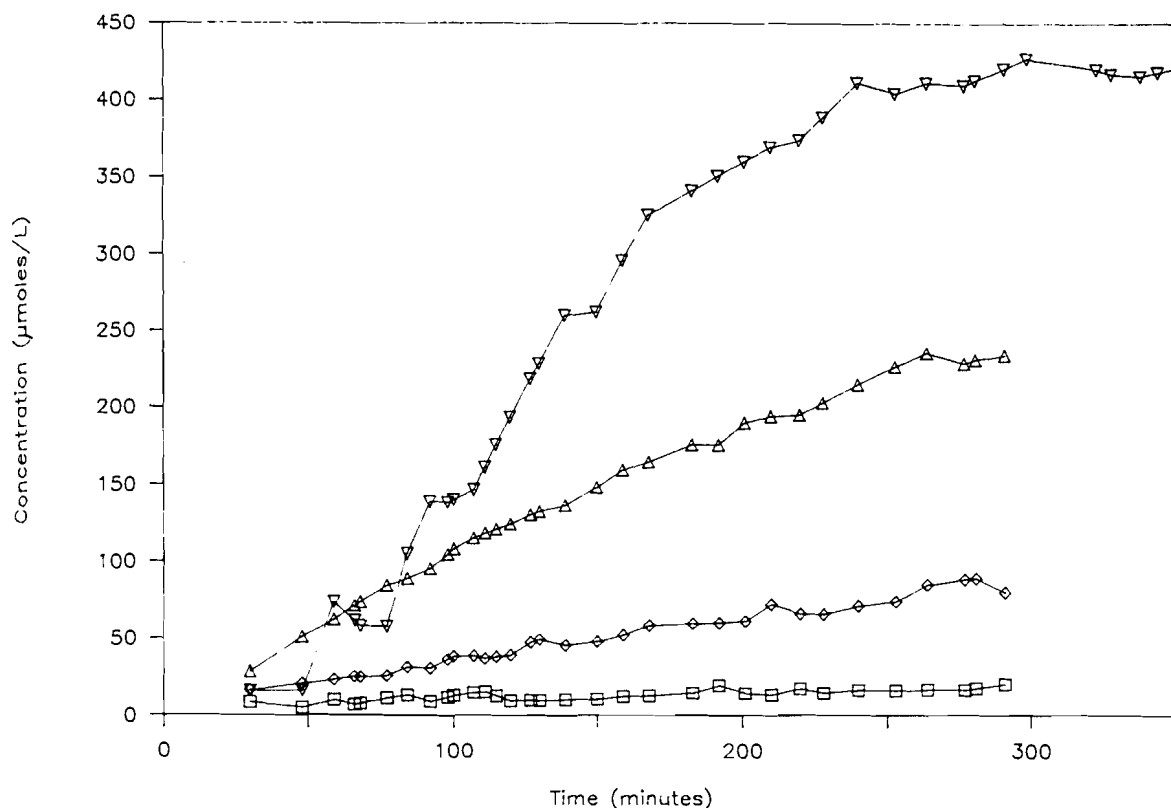


Figure 2. Time course of acetoacetate conversion to acetone, incubated at 45°C (□----□), 55°C (◇----◇), 65°C (Δ----Δ), and 90°C (▽----▽).

increase the total recovery (Figure 2). A time course study of different concentrations of acetoacetate in aqueous solutions, at 90°C, confirmed that conversion of acetoacetate to acetone was complete in 250 minutes (Figure 3). The maximum percent recovery at concentrations above 100 μmol/L was approximately 85% (Figure 3). In all subsequent analyses determination of acetoacetate concentration was performed by heating the sample to 90°C for 5 hours prior to headspace gas chromatography.

Recovery of acetoacetate from spiked aqueous solutions positively correlated with the expected values ( $r^2=0.9988$ ) from 20 to 500 μmol/L (Figure 4). The mean percent recovery over the entire range was 84.2% (Table 1B). Acetoacetate recovery from spiked blood samples, corrected for the presence of endogenous ketone bodies, was approximately 10% lower than the recovery from the aqueous standards, at concentrations above 100 μmol/L (Table 1B). At lower concentrations the recovery was more variable (44–115%). Furthermore, recovery of acetoacetate from the blood samples exhibited linearity throughout the entire range ( $r^2=0.9965$ ).

Heating spiked aqueous samples, in the absence of strong hydrolyzing agents, consistently converted 85% of the available acetoacetate to acetone. The apparent decreased recovery of acetoacetate from blood samples is a product of the effect of the blood proteins on the measurement of acetone rather than a net decrease in the conversion rate of acetoacetate to acetone. Recovery of acetone from spiked blood products was approximately 7% lower than recovery from aqueous samples (Table 1A). This mode of acetoacetate conversion minimizes the possibility of the production of chemical products other than acetone.

### Determination of 3-Hydroxybutyrate Concentration

Conversion of 3-hydroxybutyrate (3OH) to acetoacetate is accomplished by the addition of 3-hydroxybutyrate dehydrogenase and B-NAD<sup>+</sup> to a sample. The sample is incubated

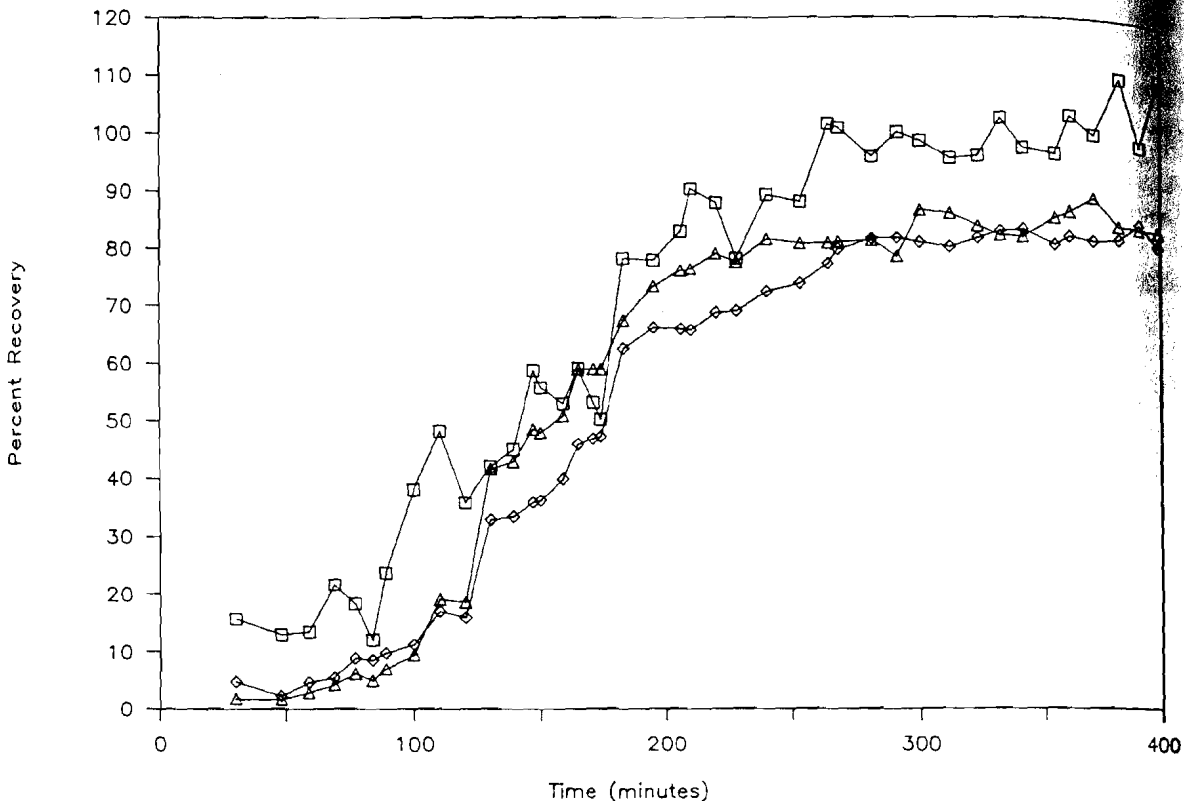


Figure 3 Time course of percent acetoacetate recovery from initial concentrations of 20  $\mu\text{mol/L}$  ( $\square$ --- $\square$ ), 150  $\mu\text{mol/L}$  ( $\Delta$ --- $\Delta$ ), and 500  $\mu\text{mol/L}$  ( $\diamond$ --- $\diamond$ ).

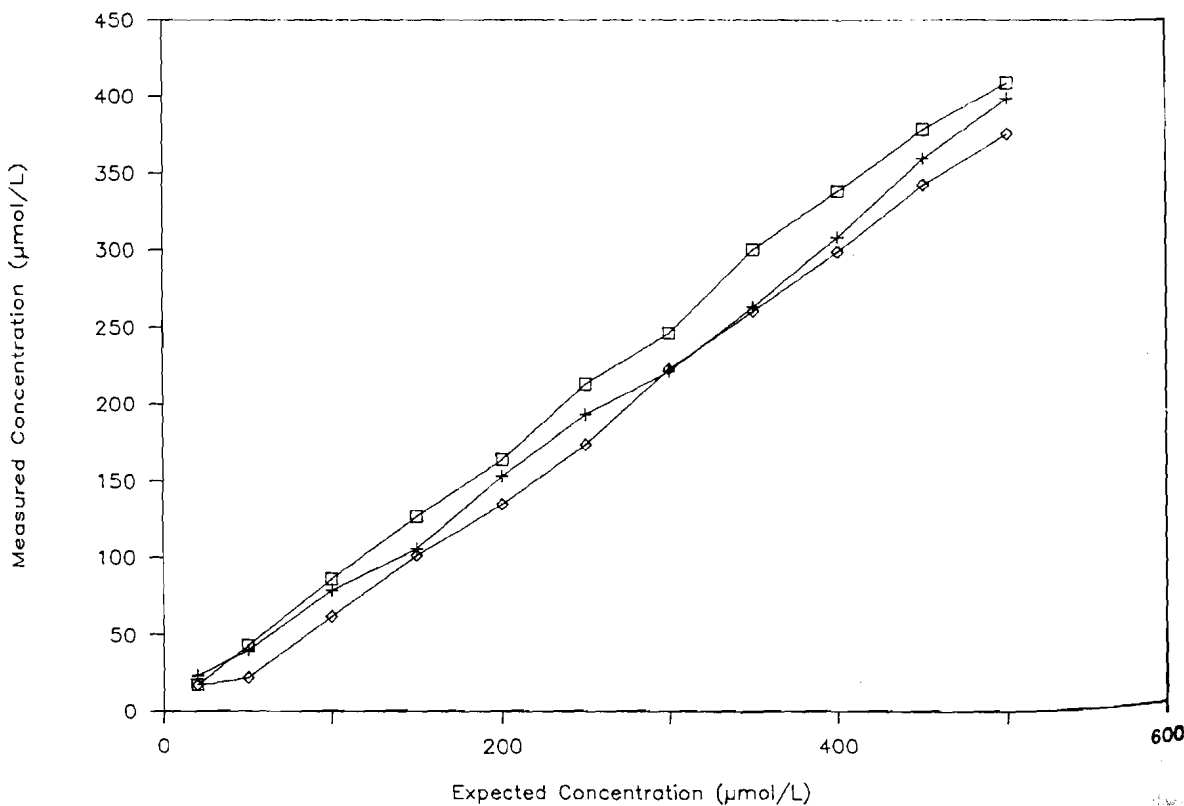


Figure 4. Acetoacetate recovery from spiked aqueous solution ( $\square$ --- $\square$ ), spiked blood A ( $\diamond$ --- $\diamond$ ), and spiked blood B (+---+) as determined by headspace gas chromatography.

at 37°C for thirty minutes during which time the enzyme converts 3OH to acetoacetate and B-NAD<sup>+</sup> is reduced to B-NADH + H<sup>+</sup>. The B-NADH produced in this reaction is reconverted to B-NAD<sup>+</sup> during the reduction of pyruvate to lactate by lactate dehydrogenase. The resulting B-NAD<sup>+</sup> can then be used to further oxidize 3OH (16).

Aqueous samples spiked with 500 μmol/L of a racemic (DL) mixture of 3OH were incubated with enzyme reagent at 37°C for 30 minutes and with no enzyme reagent. The samples were then heated to 90°C for varying periods of time, allowed to equilibrate in the automatic turntable, and the concentration of acetone determined. Maximum recovery of the spiked samples was approximately 60% after 200 minutes of heating (Figure 5). There was no recovery of 3OH by heating at 90°C in the absence of the enzyme reagent. The specificity of the enzyme for D-3OH, the 3OH stereoisomer produced as a byproduct of fatty acid oxidation, was confirmed utilizing aqueous samples that had been spiked, at several concentrations, with either the D form or L form of 3OH (Figure 6). Ninety percent of the D form was recovered at all concentrations examined. The L form was approximately 10% cross-reactive. Blood samples subjected to the same treatment can be expected to exhibit 80–90% conversion of 3OH to acetone.

### Analysis of Case Specimens

Forty-six blood samples from sexual assault cases were examined for total ketone body concentration. The blood samples were taken  $8.6 \pm 7.1$  (mean  $\pm$  SD) hours after the reported time of the assault. The ketone body analysis occurred at times up to one year after the blood was taken. The cases were divided into two groups, "visible" and "no visible" trauma, according to the police case submission form and the medical form completed at the hospital. The visible trauma group consisted of those individuals with some obvious physical injury, such as bruises, scratches, and bite marks, whereas the no visible trauma group had no visible physical injuries. Of the 46 individuals examined 13 had visible signs of injury while 33 did not. All were female.

The average total ketone body concentration for all cases was 264.4 μmol/L (Table 2). This is not significantly ( $p > 0.5$ ) higher than the mean total ketone body concentration for the control bloods (213.3 μmol/L). Removal of case samples that contained ethanol, to more closely match the condition of the control samples, resulted in an average total ketone body concentration of 310.1 μmol/L among the sexual assault victims (Table 3). This was significantly higher than the control values ( $P < 0.05$ ) and in agreement with

TABLE 2  
KETONE BODY DISTRIBUTION IN THE BLOOD OF VICTIMS OF SEXUAL ASSAULT

	Acetone	Acetoacetate	3-OH-butyrate	Total
Controls	43.1 <sup>1</sup> (13.9–72.2) <sup>2</sup>	61.0 (39.3–100.2)	109.3 (79.7–149.2)	213.3 (174.1–231.3)
All Cases	79.4 (1.7–372.7)	29.6 (0–185)	158.9 (43.3–705.2)	264.4 (76.8–1118)
Visible Trauma	63.3 (18.8–185)	37.6 (5.8–185)	125.4 (45.7–363.4)	226.9 (93.3–543.3)
No Visible Trauma	85.8 (1.7–372.7)	26.4 (0–131.8)	171.1 (43.3–705.2)	283.3 (76.8–1118)

1 – Average concentration (μmol/L).

2 – Range (μmol/L).

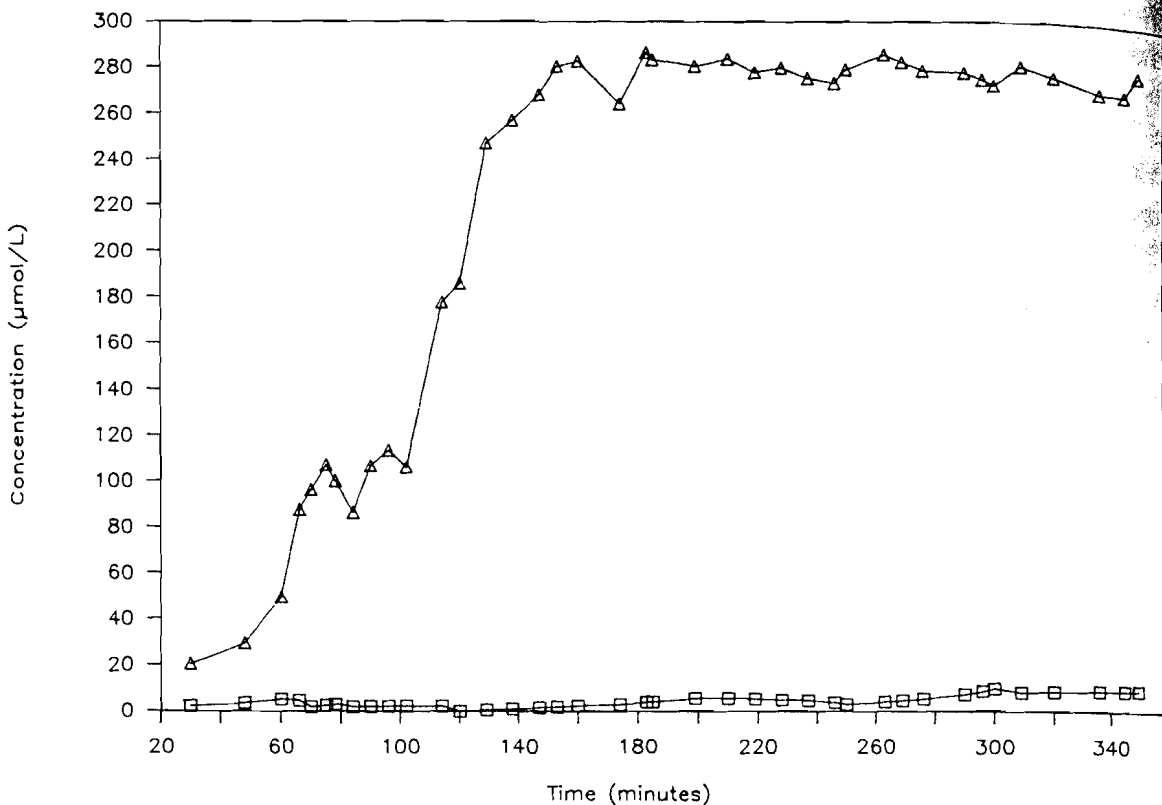


Figure 5 3-hydroxybutyrate recovery without the addition of enzyme reagent (□ --- □), and with prior incubation at 37°C for 30 minutes in the presence of the enzyme reagent (Δ --- Δ).

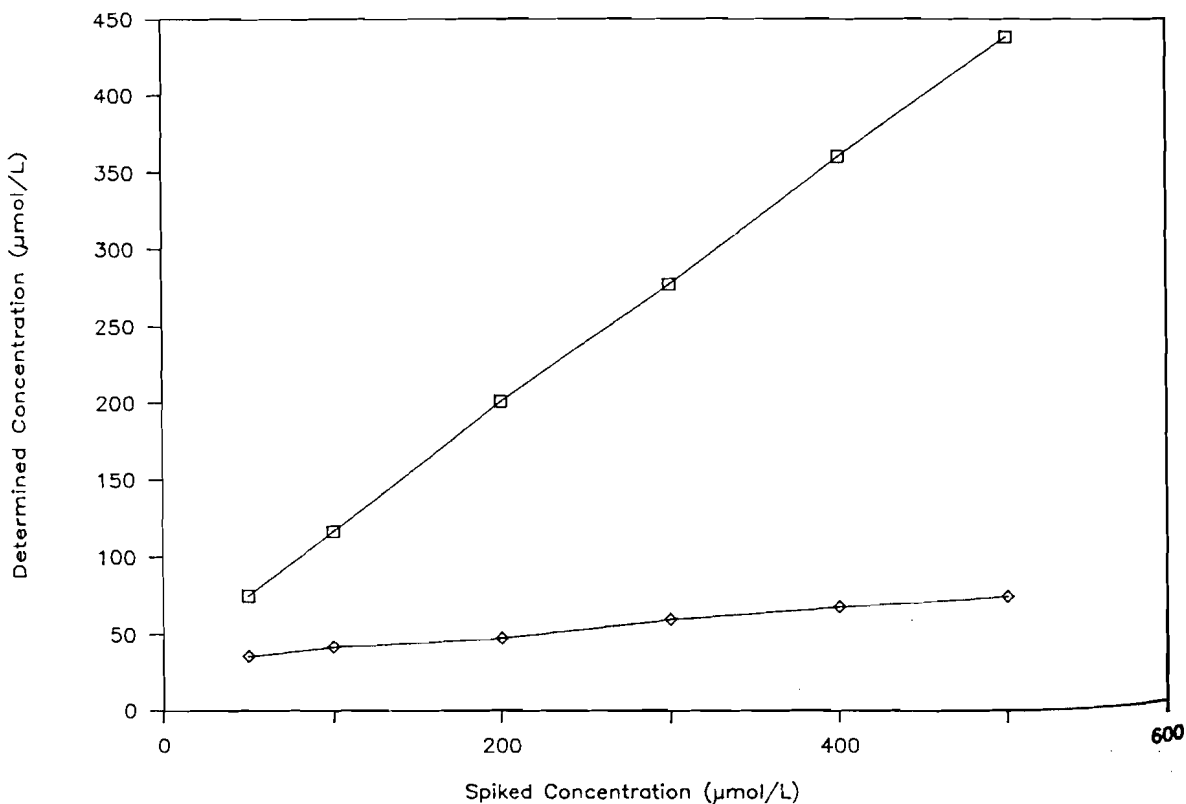


Figure 6 Enzymatic recovery of the D stereoisomer (□ --- □) and the L stereoisomer (◇ --- ◇) of 3-hydroxybutyrate from spiked aqueous standards.

previous observations from this laboratory of an increased incidence of acetone production in physically traumatized individuals (10, 11). The mean acetone concentration was higher than the mean acetoacetate concentration (Table 2). In clinical situations the plasma acetone level is always less than the blood acetoacetate level except in cases of established ketoacidosis (13). In this instance the higher levels of acetone are probably due to spontaneous, non-enzymatic decarboxylation of the acetoacetate because of the extended period of time from acquisition of the sample to analysis.

Previously we had reported, in examination of acetone levels only, that the incidence of acetone is related to the incidence of trauma, the higher incidence of acetone occurring in the visible trauma group. The current samples were separated into the visible (n=13) and no visible (n=33) trauma groups. Comparison of the total ketone body concentrations show no significant difference ( $p > 0.5$ ) between the two groups (Table 2). In this study, all cases (mean acetone concentration  $79.4 \mu\text{mol/L}$ , range, 1.7–372.7) were included in each category, while in the previous study (n=255) only those cases where the acetone concentration was greater than 1 mg/dL ( $172 \mu\text{mol/L}$ ) were included. This suggests that individuals with visible signs of trauma may have a higher incidence of very high levels of ketone bodies, but that any form of trauma is enough to elevate the total ketone body pool above control values.

Within the current study, the no visible trauma group had a higher mean acetone concentration than the visible trauma group (Table 2). This appears to be in direct conflict with the former study and may be due, in part, to the smaller sample size used in this study or to the previously reported ethanol effect (10). Examination of the two groups show that 46% (n=6) of the victims with visible trauma had ethanol in their blood as compared with 24% (n=8) of the victims with no visible trauma. Ethanol may affect ketone body expression by an indirect action on ketone body synthesis (10). The metabolism of ethanol by the liver decreases the amount of available  $\text{NAD}^+$  and produces excess amount of NADH (19, 20) which could cause a shift in ketone body production from acetone and acetoacetate to 3OH (21, 22, 23). Although ethanol consumption decreases the mean total ketone body concentration in both groups (Table 3) there is a significant difference ( $p < 0.001$ ) in the degree of decrease between the two groups. The visible trauma victims had a lower mean ketone body concentration and a narrower range of concentrations than the no visible trauma group (Table 3). The mean blood alcohol concentration of the visible trauma victims was higher (130.2 mg/dL, range 49–333) than the no visible trauma group (75.2 mg/dL, range, 17–132). This suggests that the concentration of the individual components of the ketone body pool decreases with increasing amounts of ethanol consumed.

TABLE 3

EFFECT OF ETHANOL ON TOTAL KETONE BODY PRODUCTION IN THE BLOOD OF SEXUAL ASSAULT VICTIMS WITH VISIBLE AND NO VISIBLE SIGNS OF TRAUMA

	Ethanol	No Ethanol
All Cases	181.5 <sup>1</sup> (102.9–450.2) <sup>2</sup>	310.1 (76.8–1118.7)
Visible Trauma	132.9 (102.9–168.2)	322.5 (93.3–543.3)
No Visible Trauma	219.2 (114.0–450.2)	303.8 (76.8–1118.7)

## CONCLUSIONS

Measurement of acetone concentrations in samples can be optimised by minor modifications to the headspace chromatography method used for ethanol measurement. Constituent parts of the ketone body pool can then be measured by systematic equimolar conversion of those components to acetone. Accelerated decarboxylation of acetoacetate to acetone is accomplished by heating the sample to 90°C for 5 hours. This method does not lead to the production of undesirable byproducts and can be readily utilized in any laboratory. Measurement of the 3-hydroxybutyrate involves enzymatic conversion to acetoacetate and subsequent temperature conversion to acetone. Specific, reproducible results are obtained and can be used to determine the relative ratios and absolute concentrations of the three ketone bodies.

Victims of sexual assault react to the stress of the assault by fatty acid mobilization and subsequent ketone body production. The average ketone body concentration for all alcohol free cases was higher (310.1  $\mu\text{mol/L}$ ) than the mean total for control individuals (213.3  $\mu\text{mol/L}$ ). The acetone:acetoacetate ratio in samples of all cases was greater than 1.0 indicating that spontaneous, non-enzymatic decarboxylation of the acetoacetate to acetone may have occurred because of the extended period of time from the actual taking of the blood sample to the time of analysis. There was no significant difference in the total ketone body concentration between the sexual assault victims that had visible signs of injury and those that did not ( $p > 0.5$ ). Alcohol consumption decreased the mean ketone body concentration in both groups. All individual components of the pool were affected suggesting that the previously observed effects of ethanol (10) are not a result of conversion of acetoacetate to 3-hydroxybutyrate, but may be the result of an interaction with some earlier stage of fatty acid metabolism. Headspace gas chromatographic determination of the total ketone body pool in blood may be of assistance in forensic interpretation of the sequence of events leading to physical assault (10) or death (11).

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