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Note

Gas chromatographic head-space analysis of clinically interesting ketone bodies

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The estimation of ketone bodies in biological fluids is an important part of the clinical and preclinical diagnosis of energy-metabolism disturbances in animals and man. Clinically, the term ketone bodies encompasses acetone, acetoacetic acid, isopropanol and 3-hydroxybutyric acid. Acetone and acetoacetic acid may be clinically termed oxidized ketone bodies and their reduced forms isopropanol and 3-hydroxybutyric acid (hydroxy-compounds) similarly termed reduced ketone bodies. The sum of oxidized and reduced ketone bodies is then termed total ketone bodies.

To prove levels of oxidized ketone bodies the classical colour reaction with nitroprusside [1] known as Legal-probe is still widely used. More recently, acetoacetic and 3-hydroxybutyric acids have been determined photometrically [2, 3] or fluorimetrically [4] after their mutual enzymic conversion. The most frequently used determination of free acetone is the photometric one [5—8]. The volatility of isopropanol and acetone, to which all other ketone bodies mentioned may be converted in a relatively simple manner, has facilitated their succesful determination by several workers [9—14] using gas chromatography. The head-space technique of analysis [15—17] has provided a further improvement on the methods available for the solution of this problem [10, 12, 18].

In this research note we have given the conditions under which we were successful in the application of gas chromatographic head-space analysis to the expeditious control of ketone-body levels in a broad range of cattle body fluids.

MATERIALS AND METHODS

As acid standards, we used a 1 *M* solution of ethylacetoacetate in 5 *M* KOH, stored at 4°, and the sodium salt of DL-3-hydroxybutyric acid (BDH, Poole, Great Britain). For analysis, there were three proven reagents: (i) alkaline reagent, 5 *M* KOH with 2 m*M* butanone as internal standard; (ii) acidic reagent, 5 *M* H_3PO_4 with 2 m*M* butanone; (iii) oxidative reagent, 0.1 *M* $K_2Cr_2O_7$ in 5 *M* H_3PO_4. We recommend storage of the reagents with butanone at room temperature as reproducibility of the results deteriorates with the use of cooled reagents. The acetone standard solutions stored at 4° are not stable after one week.

In separate stages of the analysis the following were pipetted:

Free acetone: 0.1 ml of sample without deproteination, plus 0.1 ml of alkaline reagent, equilibration for 15 min before GC analysis.

Oxidized ketone bodies: the same procedure, with acidic reagent replacing the alkaline one.

Total ketone bodies: 0.1 ml of sample without deproteination plus 0.1 ml of acidic reagent, heated in a boiling water bath for 3—5 min; after cooling to room temperature 0.5 ml of oxidative reagent is added as rapidly as possible through the septum, using a polypropylene syringe; the bottle set is immediately returned to the boiling water bath for 40 min and equilibrated for 15 min before GC analysis.

Samples and reagents were dosed with a micropipette into 15-ml dry test-bottles (biochemical test-bottles e.g. by Boehringer, Mannheim, G.F.R.) to which approximately 0.5 g of crystalline potassium sulfate had previously been added. The sets of bottles capped with rubber septa and perforated covering-stoppers were equilibrated in a thermostated water bath (70°). Samples of 1.5 ml of vapour were taken for analysis using a water-jacketed and temperature-controlled (70°) 2-ml injection syringe (Fig. 1) and injected into

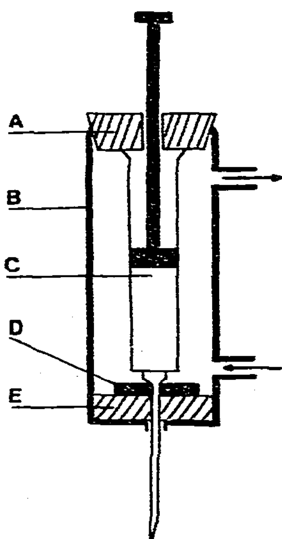

Fig. 1. Sampling device: A = rubber stopper, B = glass thermostating jacket, C = injection syringe, D = metal ring, E = rubber septum.

the gas chromatograph (CHROM 4, Laboratory Equipment, Prague, Czechoslovakia). Flame-ionization detection was used and experimental conditions were as follows: glass column 120 × 0.3 I.D.; Porapak R resin (100—120 mesh); column temperature, 180°; injector temperature 185°; carrier gas, nitrogen; flow-rate, 57 ml/min. The retention time of butanone was 3.2 min and the retention ratio of acetone 0.47. For quantitation, the peak-height ratios of acetone—butanone were used following prior confirmation of the full acetone recovery by the standard-additions method [19]. The calibration curve was without a blank value (passed the zero) and was linear at least to a 100 m*M* concentration of acetone. This limit includes all practical clinical cases.

RESULTS AND DISCUSSION

The method for the determination of physiological ketone-body levels has to be extremely sensitive. Higher sensitivity of the gas chromatographic head-space analysis may be achieved through increasing the acetone vapour tension by using a higher temperature (70°) and by the addition of an inorganic salt (K_2SO_4) to the reaction mixture. The salt simultaneously suppresses the influence of different matrices (sample composition). During the determination of total ketone bodies the sulfate must not be completely dissolved at 100° as the conversion of 3-hydroxybutyric acid to acetone would be drastically reduced. The sulfate addition is advisable particularly for high-protein samples which may be reproducibly, analyzed without deproteination. Variations in the amount of sulfate from 0.5 to 1.6 g do not influence the results, therefore weighing is unnecessary. Addition of K_2SO_4 is also desirable if the standard addition method [17, 19] is used, the results of which are, by nature, independent of matrix differences.

The optimal reagent compositions were found, by trial and error, from wide ranges of component concentrations. The alkaline reagent ensures sufficient stability of acetoacetic acid for at least an hour during which time series of 12—14 samples may be analyzed. The acidic reagent with phosphoric acid breaks the sample buffer system and decreases the pH to a point where acetoacetic acid decarboxylation is rapid and quantitative at equilibration temperature and there is no risk of conversion of the 3-hydroxybutyric acid to crotonic acid [5]. Sulfuric acid works more slowly and the samples must be heated in a boiling water bath. Consequently, for the determination of total ketone bodies, the decarboxylation step must be performed before the addition of oxidative reagent in order to prevent decomposition mechanisms other than that to acetone (loss of about 90% of acetoacetic acid) [20].

The composition of the acidic and oxidative reagents ensures quantitative conversion of reduced ketone bodies to acetone. Older methods [22] have used exclusively the dichromate—sulfuric acid mixtures, compositions of which were determined using the 3-hydroxybutyric acid standard solutions. But the degree of conversion was only acceptable for a relatively low and narrow concentration range of sulfuric acid and dichromate, and could be incorrectly, applied to the biological samples. A higher amount of eaily-oxidizable compounds decreases the oxidative capacity of the reagent substantially and the

composition of the reaction mixture leaves the optimal range. As a consequence, the actual conversion of the ketone bodies to be estimated decrease and the use of previously estimated standard factors leads to lower results. The most expressive decrease was found with milk samples. The oxidative reagent with phosphoric acid fully converts the ketone bodies present both in the standard and the milk sample. The conversion using an analogous reagent with sulfuric acid produces the value of 75% for the standard and about 50% for the milk sample.

Further, the parameter of time must be taken into consideration. After addition of oxidative reagent to the sample it is necessary to heat the reaction mixture as quickly as possible in a boiling water bath. At room temperature, 3-hydroxybutyric acid decomposes to products other than acetone [20, 21]. A long contact (days) with acidic oxidant causes loss of ketones probably due to complex-formation [20]. Both effects are much more evident in the case of oxidative reagent with sulfuric rather than phosphoric acid.

Vapour sampling and injection with the described device is simple and with the use of internal standard also surprisingly precise. Duplicate vapour sampling from the same bottle gives only negligible differences between the results of the first and second analyses. The syringe may be inserted into the bottle empty or a volume of air, equal to the volume of vapour withdrawn [10, 17] may be added, but the chosen procedure must be consistent.

The use of butanone as internal standard is fully justified in the case of human samples [14], but its presence in the body fluids of other species always has to be verified. Table I contains the natural butanone concentrations found in some stages of the body-fluid analyses of healthy and ketotic late-pregnancy cows together with the values for the relative increase of the total butanone concentration over the 2 m*M* butanone used as internal standard. At coincident stages of analysis, the calibration factors obtained with standard solutions have to be divided by the appropriate value for the relative increase in order to correct for the natural butanone background. It is true that this background changes slightly in cases of ketosis but the changes are negligible for our purpose [20].

TABLE I

NATURAL BUTANONE CONCENTRATIONS AND THE VALUES OF RELATIVE INCREASE OF THE TOTAL BUTANONE CONCENTRATIONS OVER THE 2 m*M* OF INTERNAL STANDARD

Sample	Number of samples	Determined ketone bodies	Natural butanone m*M* ($\bar{x}$ ± S.D.)	Relative increase
Urine	24	Oxidized	0.078±0.017	1.04
Urine	24	Total	0.38 ±0.05	1.19
Blood plasma	36	Total	0.133±0.008	1.065
Amniotic fluid	18	Total	0.053±0.007	1.025
Allantoic fluid	20	Total	0.087±0.007	1.04
Foetal serum	18	Total	0.117±0.007	1.06
Foetal urine	12	Total	0.052±0.008	1.025

A serious problem is caused by the presence of additional compounds which can decompose to acetone or other interfering substances. Among these belong especially glycides (glucose, fructose, lactose), lactate and disinfectants. The strongest interference occurs with alkaline reagent, as these compounds then give acetone values almost comparable to physiological levels [20]. This problem cannot be solved by subtraction of the correction factor as samples anticipated to contain high amounts of glycide (e.g. foetal fluids) often give acetone values much lower than the corresponding glycide standard and vice versa. Samples without provable amounts of glycide (urine) sometimes give higher values for free acetone than for oxidized ketone bodies. We found that the use of acidic and oxidative reagents is almost free from this interference. As the determination of free acetone is of questionable diagnostic value [23] we recommend, for current diagnostic analyses, the determination of oxidized and total ketone bodies only. The interference of disinfectants is rare and often may be overcome by changing the analytical conditions [18].

The presented method permits precise determination of total and/or reduced ketone-body amounts. Older methods converted isopropanol and 3-hydroxybutyric acid to the acetone in different degrees and the precise calculation was not possible unless the separate determination of isopropanol was made [24]. With our procedure, the reproducibility expressed as coefficient of variation of a six-times-repeated analysis of the same sample is better than 2.5% for both standard and biological samples. This precision, together with the total conversion of the reduced ketone bodies to acetone, is especially valuable in the case of analyses of non-traditional samples (milk, foetal fluids) which were poorly reproducible using older methods.

In Table II are given some physiological levels of oxidized and total ketone bodies in body fluids of late-pregnancy cows.

Besides direct measurements of the concentrations of ketone bodies, the ratio of oxidized to total ketone bodies concentrations (O:T) expressed as a percentage may be of greater diagnostic value. This ratio is more sensitive and rapidly exceeds the physiological range during bovine ketosis [20, 23].

TABLE II

PHYSIOLOGICAL LEVELS OF OXIDIZED AND TOTAL KETONE BODIES IN BODY FLUIDS OF LATE-PREGNANCY COWS

Sample	Number of samples	Ketone bodies mM ($\bar{x}\pm$S.D.)	
		Oxidized	Total
Urine	7	0.039±0.007	1.01±0.16
Blood plasma	9	0.063±0.016	1.23±0.16
Amniotic fluid	8	0.034±0.006	0.33±0.03
Allantoic fluid	9	0.030±0.005	1.06±0.10
Foetal serum	8	0.023±0.002	0.64±0.06
Foetal urine	4	0.028±0.009	0.55±0.17

CONCLUSIONS

For ketone-body determination, gas chromatographic head-space analysis was used successfully. The matrix effect of different biological samples was suppressed by addition of crystalline potassium sulfate together with the use of concentrated reagents and internal standard. The optimized reagents comprised 5 *M* KOH, 5 *M* H_3PO_4, and 0.1 *M* $K_2Cr_2O_7$ in 5 *M* H_3PO_4. As the internal standard 2 m*M* butanone was used.

For determination of oxidized and total ketone bodies 0.2 ml of the biological sample without deproteination is sufficient. Reproducibility of the method is better than 2.5% and stoichiometric conversion of isopropanol, 3-hydroxybutyric and acetoacetic acids to acetone and total recovery of acetone is reached with all samples mentioned. The method was succesfully verified in serial analyses and is suitable for diagnosis of ketotic states in animals and man. The mean physiological values for different body fluids of late pregnancy cows are given.

REFERENCES

1 H.J. Roth and K.H. Surborg, Arch. Pharm. (Weinheim), 301 (1968) 686.
2 C. Remesy and C. Demigne, Ann. Rech. Vétér., 7 (1976) 329.
3 D.H. Williamson, J. Mellanby and H.A. Krebs, Biochem. J., 82 (1962) 90.
4 C. Olsen, Clin. Chim. Acta, 33 (1971) 293.
5 H. Steger and J. Voigt, Arch. Tierernähr., 20 (1970) 631.
6 H. Göschke, Clin. Chim. Acta, 28 (1970) 359.
7 H.G. Schuster and G. Baasch, Z. Med. Labortechn., 12 (1971) 312.
8 B. Majewska and J. Rybczyńska, Med. Wet., 32 (1976) 439.
9 M.J. Sulway and J.M. Malins, Lancet, ii (1970) 736.
10 G.J. van Stekelenburg and J.W. de Bruyn, Clin. Chim. Acta, 28 (1970) 233.
11 M.D. Trotter, M.J. Sulway and E. Trotter, Clin. Chim. Acta, 35 (1971) 137.
12 C.J.P. Eriksson, Anal. Biochem., 47 (1972) 235.
13 I.A. McDonald, L.P. Hackett and L.J. Dusci, Clin. Chim. Acta, 63 (1975) 235.
14 L. Siegel, N.I. Robin and L.J. McDonald, Clin. Chem., 23 (1977) 46.
15 H. Hachenberg and A.P. Schmidt, Gas Chromatographic Headspace Analysis, Heyden and Son, London, 1977.
16 S. Özeris and R. Bassette, Anal. Chem., 35 (1963) 1091.
17 J. Drozd and J. Novák, J. Chromatogr., 136 (1977) 37.
18 G.J. van Stekelenburg and G. Koorevaar, Clin. Chim. Acta, 34 (1971) 305.
19 J. Novák, Quantitative Analysis by Gas Chromatography, Marcel Dekker, New York, 1975.
20 P. Hradecký, Thesis, Faculty of Natural Sciences, J.E. Purkyně University, Brno, 1978.
21 D.D. Van Slyke, J. Biol. Chem., 32 (1917) 455.
22 T.E. Weichselbaum and M. Somogyi, J. Biol. Chem., 140 (1941) 5.
23 P. Hradecký, P. Jágoš, E. Kudláč and J. Janák, Brit. Vet. J., submitted for publication.
24 C. Thin and A. Robertson, Biochem. J., 51 (1952) 218.