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High-performance liquid chromatographic determination of acetone in blood and urine in the clinical diagnostic laboratory

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ABSTRACT

A method for the determination of acetone in plasma or urine by high-performance liquid chromatography (HPLC) was developed. Plasma specimens are deproteinized with acetonitrile (1:1, v/v); 2,4-dinitrophenylhydrazine (DNPH) is added to the supernatant or to filtered urine samples, similarly treated with acetonitrile (2:1, v/v) to prevent crystallization of the synthesized phenylhydrazone. An aliquot (20 μl) of the reaction mixture was subjected to HPLC at ambient temperature using a reversed-phase Pecosphere 3×3 C_{18} column with acetonitrile–water (45:55, v/v) as eluent at a flow-rate of 1 ml/min and detection at 365 nm. Hydroxyacetone and acetoacetate phenylhydrazone derivatives do not interfere. The identification of acetone by its retention time was confirmed by comparison with a laboratory-synthesized acetone DNPH derivative. The concentration of acetone, eluted within 3 min, was determined by the peak-height method. The detection limit was 0.034 mmol/l; the relative standard deviations were $< 5\%$ within run ($n = 20$) and $< 10\%$ between run ($n = 20$).

INTRODUCTION

In conditions that limit carbohydrate utilization with a resulting increase in fat utilization (diabetes mellitus, starvation, alcoholism), ketone bodies (acetone, acetoacetate and β-hydroxybutyrate) are produced by the liver [1–4]. Acetone, which is formed spontaneously from acetoacetate by irreversible decarboxylation, seems to play a minor role, but some reports over the last 10 years have indicated a renewed interest in its metabolism and there is a need for a method for its measurement, free from interferences [5]. Moreover, acetone has recently become recognized in plasma [6] and especially in urine [7,8] as an indicator of occupational exposure to acetone or isopropanol [9]. The exposure to acetone is evaluated by measurement of the ketone itself in urine in which the ketone pressure is in equilibrium with the pressure in the blood and alveoli [7,8]. In the biological monitoring of exposure to isopropanol, the urinary acetone is present as its catabolite.

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Chemical methods used to test acetone often lack in specificity [10,11]. Enzymatic methods are more specific but more complex and have long assay times and gas chromatographic methods, although widely used, are applied with difficulty as routine tests [12–20]. Here we describe a rapid and simple high-performance liquid chromatographic (HPLC) procedure that can be used for the routine measurement of acetone in biological fluids, such as plasma and urine. The method is based on the derivatization of acetone with 2,4-dinitrophenylhydrazine (DNPH), using very small amounts of sample; the derivative concentration is rapidly obtained without recourse to a solvent extraction step. Reference ranges in plasma and urine for healthy subjects were determined and data obtained for diabetic subjects and exposed workers are reported.

EXPERIMENTAL

Reagents and solutions

All reagents were of analytical-reagent grade. Deionized water was distilled in an all-glass still. The following chemicals were obtained from Sigma (St. Louis, MO, U.S.A.): hydroxyacetone, sodium acetoacetate, DNPH and acetonitrile. Acetone and hydrochloric acid were obtained from Aldrich (Milwaukee, WI, U.S.A.).

DNPH reagent. A 0.25-g amount of DNPH was dissolved in a hydrochloric acid–distilled water (40:60, v/v) mixture by warming in a water-bath at 60°C for 20 min.

Stock solution of acetone DNPH derivative. The phenylhydrazone was synthesized in crystalline form from a mixture of a 10% aqueous acetone solution and DNPH reagent (75:25, v/v). The crystalline product, separated by centrifugation (1800 *g* for 15 min) was washed twice with deionized water and recrystallized from ethanol as described elsewhere [21]. The DNPH derivative was dissolved in acetonitrile and, by injection into the HPLC system, was utilized for retention time control.

Working standard solutions. Calibration graphs were prepared by adding known amounts of acetone (0.107, 0.215, 0.430, 0.860, 1.720 and 3.440 mmol/l) to plasma or urine specimens of healthy unexposed men.

Sample collection and preparation

Blood was collected, with K_2EDTA as anticoagulating agent, from diabetic patients, workers exposed to acetone and healthy unexposed men. As soon as possible after collection, the plasma was separated in a refrigerated (4°C) centrifuge (1000 *g* for 5 min) and either analysed immediately or stored at −20°C to minimize decarboxylation of acetoacetate and loss of acetone and thawed at 4°C just before analysis.

Urine specimens, kept in well closed bottles, were obtained from healthy unexposed men and from workers exposed to acetone. The samples, filtered through 0.2-μm poresize disposable filters (Millepore, Milford, MA, U.S.A.), were stored at 4°C for 48 h or at −20°C for longer periods.

Volumes 200 μl of each plasma sample or standard were added to 1.5-ml centrifuge tubes containing 200 μl of acetonitrile and the tubes were stoppered, vortex mixed (5 s) and centrifuged at 4°C (1000 *g* for 5 min). A 200-μl volume of the supernatant was transferred to another tube and 40 μl of DNPH reagent were added. The mixture was vortex mixed for 5 s and, after 5 min, 20-μl aliquots were injected at room temperature into the HPLC system and the peak heights read at 365 nm.

A volume of 500 μl of each filtered urine sample or standard was added to a tube containing 250 μl of acetonitrile and 200 μl of DNPH reagent. The mixture was vortex mixed for 5 s and, after 5 min, 20-μl aliquots were used for chromatography.

Chromatography

HPLC separation and peak detection of acetone DNPH derivative were carried out on a Pecosphere 3 × 3 C_{18} (3 μm packing) reversed-phase column (3.3 cm × 4.6 mm I.D.) in an HPLC system consisting of a Series 410 LC pump and an LC 90 UV ultraviolet spectrophotometer (both from Perkin-Elmer, Norwalk, CT, U.S.A.), coupled to an integrator developed by S.P.E. Sistemi e Progetti Elettronici (Brescia, Italy). The mobile phase was acetonitrile–water mixture (45:55, v/v) at a flow-rate of 1.0 ml/min.

Statistics

The within-days relative standard deviation (R.S.D.) was based on the analysis of twenty samples prepared from a pooled specimen of plasma or urine, all samples being extracted and analysed during the same day. The between-days R.S.D. was based on the analysis of single samples obtained from the same plasma or urine pool (stored at −20°C and thawed at 4°C just before acetone determination) on 20 separate days. Calibration graphs were prepared for each between-day analysis. Single injections of samples and standards were made. The means and standard deviations were calculated to determine the within-day and between-day R.S.D.s.

RESULTS

Fig. 1 shows chromatograms of (a) a plasma sample and (b) a urine sample from an exposed subject. Hydroxyacetone and acetoacetate DNPH derivatives do not interfere; the first derivative has a retention time of 57 s (Fig. 2) and the peak of the second derivative is not eluted under the chromatographic conditions described.

The identity of the acetone DNPH derivative peak was confirmed by the retention time of a laboratory-synthesized acetone DNPH derivative dissolved in acetonitrile. The calibration graphs (obtained by adding known amounts of acetone) were linear for both plasma and urine up to at least 3.440 mmol/l and were represented by the regression equations $y = 1.97 + 123.8x$ for plasma and $y = 0.366 + 66.3x$ for urine, where y = peak height (mV) and x = acetone concentration (mmol/l). The lower sensitivity in the urine is due to the presence of other ketonic and aldehydic groups which compete with acetone for DNPH. The addition of more DNPH or dilution of the urine did not improve the sensitivity.

The limit of detection in both plasma and urine at a signal-to-noise ratio of 2 was 0.034 mmol/l. The within-run and between-run R.S.D.s were 3.8% and 7.4%, respectively, for plasma and 4.8% and 9.2%, respectively, for urine ($n = 20$), using a pooled plasma and urine specimen containing 0.172 mmol/l of acetone. Instrumental variation accounted for about 2% of the total variation.

The reference acetone values [$x \pm 2\ s$ (s = standard deviation)] for healthy unexposed men were 0.034–0.120 mmol/l with a mean value of 0.075 mmol/l in plasma ($n = 20$) and 0.034–0.095 mmol/l with a mean value of 0.052 mmol/l in urine ($n = 20$). These compare well with the reference values suggested by other workers and obtained by gas chromatographic methods [22,23].

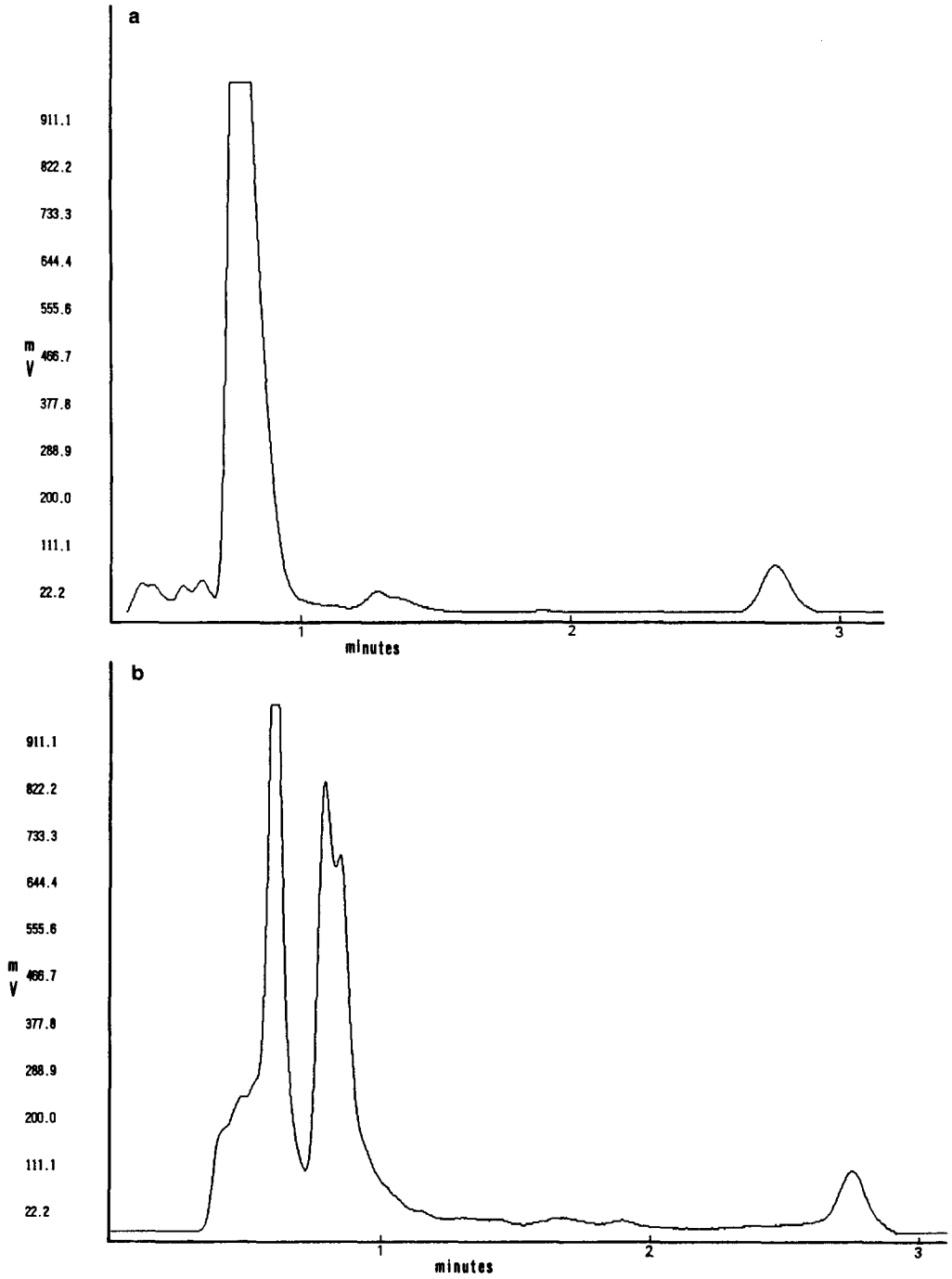

Fig. 1. HPLC of acetone DNPH derivative (a) in plasma and (b) in urine samples (retention time, 2 min 44 s).

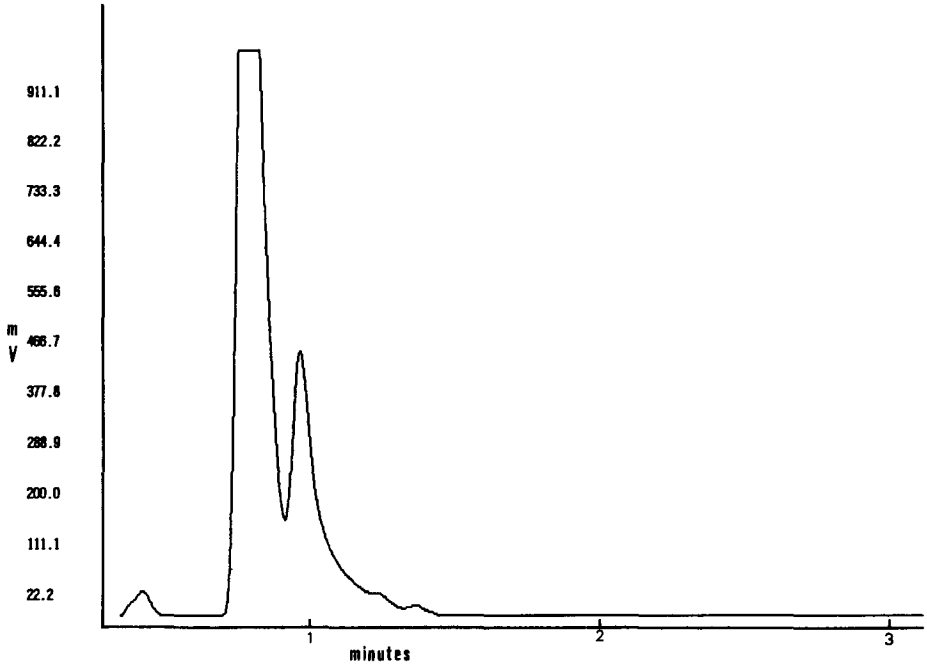

Fig. 2. HPLC of hydroxyacetone DNPH derivative (retention time, 57 s).

In the biological monitoring of workers exposed to acetone at the end of a shift and at the end of the working week, the acetone concentrations were 0.035–0.170 mmol/l with a mean value of 0.106 mmol/l in plasma and 0.103–1.29 mmol/l with a mean value of 0.320 mmol/l in urine. The determined concentrations were lower than the biological exposure indices given by different workers [22,24,25].

The range of acetone levels in plasma from two different classes of diabetic subjects (20 patients cured by diet with glucose values lower than 8.34 mmol/l and 30 patients with type I insulin-dependent diabetes mellitus on therapy with insulin) was 0.040–0.160 mmol/l with a mean value of 0.100 mmol/l.

DISCUSSION

The HPLC method described here overcomes many of the problems in the determination of acetone in biological fluids and the preanalytical errors. The volatile ketone is promptly stabilized by conversion into its DNPH derivative and rapidly determined without recourse to a solvent extraction step. If we wish to examine more samples in one chromatographic session, it is possible to minimize decarboxylation of acetoacetate and loss of acetone by preserving the samples at $-20°C$ and thawing them at 4°C just before actone determination [14]. The added acetonitrile deproteinizes the plasma and simultaneously prevents the crystallization of the easily and rapidly formed DNPH derivative. The determination is free of interference from hydroxyacetone [26] and acetoacetate. The proposed method possesses the necessary sensitivity and shows excellent reproducibility. Up to 100 samples can be prepared in less than

30 min and individual chromatographic results obtained in less than 3 min. The method uses very inexpensive reagents. The proposed HPLC method can therefore be used to great advantage over current gas chromatographic methods; it can be used in experiments requiring multiple samples and specific activity determination for the routine measurement of acetone in diabetic patients and in biological monitoring of exposed workers. The use of very small sample amounts makes this a favourable method in paediatrics.

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