Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 7, pp. 843–850, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00

Determination of acetoacetate in urine by solid-phase spectrophotometry

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Abstract: A method for the determination of acetoacetate has been developed based on solid-phase spectrophotometry (SPS). The acetoacetate reacts with nitroprusside and glycine and the reaction product is sorbed on Dowex 1-X8 resin. The absorbance of the resin phase at 590 and 720 nm is measured directly. The calibration graph is linear up to 3.3 mg l^{-1} and the RSD is 1.9%. The detection limit is 7.6 μg l^{-1}. The method has been applied to the determination of acetoacetate in normal and diabetic subjects' urine without pretreatment of the samples, and the results compared with those of ^{1}H-NMR and homogeneous nitroprusside methods.

Keywords: *Acetoacetate determination; nitroprusside reaction; solid-phase spectrophotometry.*

Introduction

Determination of urine and serum levels of ketone bodies is clinically important. The most common methods used for the estimation of acetoacetate involve its reaction with nitroprusside. This reaction is used for the routine semiquantitative estimation of acetoacetate in commercially available dipstick- and tablet-type systems. It is also described for spectrophotometric determination in homogeneous solutions [1].

The probable nature of reaction products has scarcely been studied. One interpretation suggests that the NO group of the nitroprusside reacts with the ketone to produce isonitrosoketone, which remains in the complex anion, while, at the same time, the iron is reduced [2]. Laios *et al.* [3] suggest a more complex mechanism extended to ketoacid. This multi-step process involves the formation of first, a ketimine which reacts further to form an enamine. The coloured product results from the reaction of nitroprusside with the enamine intermediate. The reaction product, however, has not been identified.

The use of this reaction for quantitation of ketone bodies presents problems resulting from the insufficient stability of the reaction product and from the complex kinetics of this reaction [3].

Our aim is to combine this common reaction for acetoacetate using the solid-phase spectrophotometry (SPS) technique in order to propose a method that resolves the

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aforementioned problems. This technique (based on the preconcentration of a charged species on an ion exchanger and the subsequent measurement of the absorbance of the species in the resin phase) has been used for the analysis of inorganic species in water [4, 5], yet scarcely used for the analysis of organic compounds [6].

SPS methods are both selective and sensitive given the selective pre-concentration effect occurring simultaneously with colour development in the resin phase, and the possibility of reducing interferences by sample dilution.

Experimental

Reagents

All chemicals used were of analytical grade; the water was doubly distilled and all experiments were carried out at room temperature.

Ion exchange resins. Dowex 1-X8 (100–200 and 200–400 mesh) anion exchange resins (Fluka Co.) were used in the chloride form. They were properly conditioned, then air-dried and stored in polyethylene containers.

Sodium nitroprusside solution, 1.91×10^{-2}M. From $Na_2[Fe(NO)(CN)_5]\cdot 2H_2O$ (Merck Co.) was prepared daily and stored at room temperature in dark bottles to avoid photochemical reaction.

Acetoacetate standard solution, 0.231 M. Stock solution was prepared by hydrolysis of ethylacetoacetate (Sigma Co.). 3 ml of doubly-distilled ethylacetoacetate were diluted with 0.29 M NaOH and allowed to stand in a refrigerator at 4°C for 48 h. The ^{1}H-NMR spectroscopy was used to check the residual ester. The signal/noise ratio calculated from a 177 scan spectrum of the hydrolysis product indicated that the ethyl acetoacetate concentration in the hydrolysate was lower than 0.5%. However, incomplete hydrolysis was obtained with a NaOH solution of lower concentration, as was indicated in the bibliography [1, 3]. Standard solutions of lower concentration were prepared by dilution with doubly-distilled water. Stock standard was renewed monthly and the working solutions daily. All solutions were stored at 4°C.

Glycine solution 1.6 M (Carlo Erba Co.) and Na_2HPO_4 0.41 M (Merck Co.).

Sodium trimethylsilyl-[$^2H_4$]-propionate 10 mM (Aldrich Co.).

Urine specimens. Urine samples obtained from healthy adult males were pooled, filtered through a 0.45-μm membrane filter paper (Millipore) and, when necessary, frozen at −20°C until their analysis. Urine samples from diabetic subjects were obtained from local hospitals, and pooled and filtered in the same manner.

Apparatus

The apparatus used included a Spectronic 2000 Bausch & Lomb spectrophotometer with 1 mm cells, a Bruker AM-300 NMR spectrometer, an Agitaser 2000 rotating-bottle agitator, and a Crison Digit-501 pH-meter with a combined glass-saturated calomel electrode.

Absorbance measurements

The absorbance of the reaction product sorbed on the resin was measured in a 1 mm cell at 590 and 720 nm (the latter is within the range where the resin only absorbs light) against a 1 mm cell well-packed with resin equilibrated with blank solution. The net absorbance, A_{RP}, for the reaction product was obtained by: $A_{RP} = A_{590} - A_{720}$ [5].

General procedure

Twenty millilitres of 1.6 M glycine solution, 6 ml of M NaOH solution, and 2 ml of 3.8×10^{-3}M nitroprusside solution were added to a sample solution containing 0.1–3.3 mg l^{-1} of acetoacetate in the final volume, making up to 100 ml with distilled water. The solution was transferred to a 1 l. polyethylene bottle and 0.2 g of the Dowex 1-X8 (200–400 mesh) resin was added. The bottle was completely covered with aluminium foil, and the mixture was then stirred for 10 min. The coloured resin beads were then collected by filtration under suction and, with the aid of a pipette, were packed into a 1-mm cell together with a small portion of the filtrate. The cell was centrifuged for 30 s at 25 ***g***. The absorbances at 590 and at 720 nm for the sample were measured against a 1-mm cell reference, similarly packed with resin equilibrated with the blank solution containing all of the reagents except acetoacetate.

Urine procedure

For normal subjects, 1 ml of sample without previous treatment was used. For diabetic subjects, it may be necessary to use a previously diluted sample. To estimate the dilution required, a reagent strip for testing ketone bodies (Keto-Diastik) was used: if the content was higher than 150 mg l^{-1}, the urine was diluted with doubly distilled water until the next test indicated the aforementioned level. A volume of 0.25 ml was used for diabetic subjects. The analyses were performed in the same manner as in the general procedure. In all cases the standard addition method was used for calibration.

Reference procedures

^{1}H-NMR method. The high-resolution ^{1}H-NMR spectroscopy method used by Nicholson [7] and Bales [8] was applied to untreated urine samples. Measurements were made using 0.45 ml of urine where 50 μl of ^2H_2O was added to provide an internal field-frequency lock for the spectrometer. This contained sodium trimethylsilyl-[$^2H_4$]-propionate (TSP) as a combined chemical shift and concentration reference. The intense water signal was suppressed by the application of a selective secondary irradiation field at the water resonance frequency. ^{1}H resonance assignments were confirmed by standard addition of acetoacetate to urine samples. By integrating the methyl ^{1}H-NMR signals for acetoacetate at 2.28 ppm, relative to an internal standard (TSP), it was possible to quantify the urine concentration. Figure 1 shows the ^{1}H-NMR spectrum of a diabetic urine sample and the amplified analytical signals for acetoacetate and standand TSP. All spectra of urine samples were registered and amplified in the same manner.

The homogeneous nitroprusside method. The nitroprusside method in solution following the procedures and working conditions proposed by Schilke [1] was used.

Results and Discussion

Absorption spectra

The reaction product between acetoacetate and nitroprusside, in the presence of glycine shows a violet colour with an absorption maximum at 550 nm in solution. This compound is fixed into anionic resins exhibiting a violet-blue colour. The net absorption spectrum in the resin phase shows a band centred around 590 nm, whose position did not change with pH over the 7–12 range.

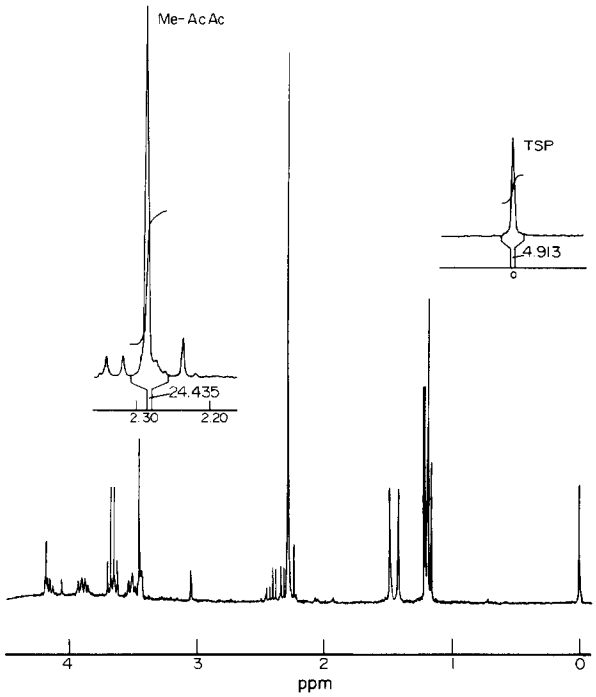

Figure 1
^{1}H-NMR spectra (300 MHz) from a diabetic urine sample; 120 scan. 0.45 ml urine diluted 1:10 with water; 50 μl of 10 mM TSP solution; [acetoacetate] added, 1.1×10^{-2} M. Enlargements: signal of methyl of acetoacetate (Me-AcAc) 2.28 ppm; signal of TSP 0.00 ppm.

Optimization of conditions

Problems with the reaction system in homogeneous solution result from the insufficient stability of the reaction product. In the method described by Shilke [1], the absorbance had to be measured at fixed times (18 min) due to the instability observed. Preliminary studies show that the colour developed on the resin phase under similar conditions, is also unstable. Optimization studies therefore were conducted in order to find a set of experimental conditions which would stabilize the system. Because most of the variables affected system stability, it was frequently necessary to study the effects of some of the variables (e.g. glycine concentration, pH and stirring time) simultaneously. To evaluate the stability, the absorbance values at appropriate time intervals were recorded.

pH dependence. No reaction was observed at pH values lower than 7. Absorbance increased with pH and was found to be the highest over the 9.5–11 pH range. However, at pH higher than 10, the instability of absorbance increased and the resin began to degrade. Hence, pH 9.2 was selected as being optimum for the standard procedure.

Influence of glycine concentration. The effect of glycine concentration was studied in the 0–1.2 M range. No reaction was observed without glycine. Absorbance increased with glycine concentration according to the equation $A = -2.03 \exp(-2.59[\text{gly}]) + 2.06 \exp(0.23[\text{gly}])$ ($r = 0.998$) and did not reach a maximum. An increase in glycine

concentration, however, causes a reduction in stability. In the standard procedure, 0.32 M was selected as a compromise between maximum sensitivity and maximum stability.

Influence of phosphate concentration. This effect was examined in the 4×10^{-3}–1×10^{-1} M range at a 8.5 pH constant. Absorbance decreased with phosphate concentration according to the equation $A = 1.39 \exp(-143.4\ [\text{phosphate}]) + 0.60 \exp(-6.42\ [\text{phosphate}])$ ($r = 0.992$). We have, therefore, suppressed the phosphate since there also is another buffer system (glycine–glycinate) in the reaction medium.

Influence of nitroprusside concentration. The influence of nitroprusside concentration is shown in Fig. 2. Optimal values to obtain maximum absorbance start for [nitroprusside]/[acetoacetate] ratios of 1 onwards. On the other hand, nitroprusside concentrations higher than 0.9 mol g^{-1} dry 200–400 mesh resin, caused resin saturation. As optimum concentration, in this study we used 7.6×10^{-5} M for acetoacetate concentrations lower than 4 mg l^{-1}.

It can be inferred from Fig. 2, that the molar ratio of nitroprusside to acetoacetate in the reaction product sorbed on the resin is 1:1.

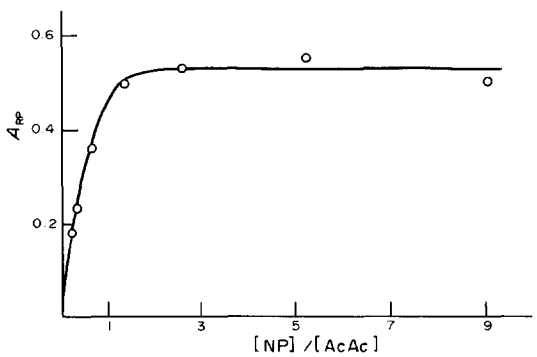

Figure 2
Influence of nitroprusside concentration. [acetoacetate], 1.4×10^{-4} M; [nitroprusside], from 2.9×10^{-5} M to 1.3×10^{-3} M; pH 8.6 (glycine 0.2 M–phosphate 0.012 M buffer); 200 mg Dowex 1 × 8 200–400 mesh resin; 100 ml sample; stirring time, 20 min.

Stirring time. Optimum stirring time was found to be 20 min for a 100–200 mesh resin, decreasing to 10 min with a 200–400 mesh resin.

Resin amount. Since the use of a large amount of resin lowers the absorbance, we used the optimum amount to fill the cell, i.e. 0.2 g, for all measurements.

Light influence. The effects of light were evaluated for two glycine concentrations (0.32 and 0.64 M), two pH values (9.0 and 9.4) and two different stirring times (5 and 10 min). The maximum absorbance was obtained when the equilibration bottles were totally covered with aluminium foil. The effect of light on absorbance change under different conditions is illustrated in Fig. 3. It is apparent, from this figure, that laboratory

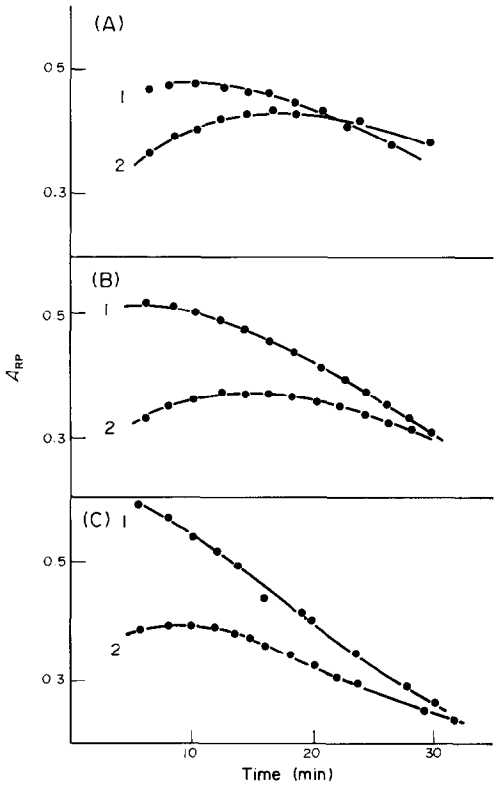

Figure 3
Stability of fixed product on the resin. (A) pH 9.0 (glycine–glycinate buffer, 0.32 M); (B) pH 9.4 (glycine–glycinate buffer, 0.32 M); (C) pH 9.0 (glycine–glycinate buffer, 0.64 M); [acetoacetate], 1.43×10^{-5} M; [nitroprusside], 7.6×10^{-5} M; 200 mg Dowex 1 × 8 200–400 mesh resin; stirring time, 10 min. 1, Protected from light; 2, without protection.

light affects reaction system kinetics. One possible explanation is the photodegradation of sodium nitroprusside [9].

The reproducibility of the determinations is consequently affected by laboratory light change; thus, the standard deviations for three data sets (five determinations) with different light intensity (daylight, halflight and protected from light) were 5.5, 3.0 and 1.9%, respectively. Hence, the bottles were protected from light in the standard procedure.

Calibration, precision, sensitivity and detection limit

The calibration graph is reasonably linear for concentrations up to 3.3 mg l^{-1} of acetoacetate for the 100-ml sample system. The equation $A_{RP} = 0.028 + 0.346\ C$ ($r = 0.9980$) holds, where C is the concentration of acetoacetate in mg l^{-1}.

Precision was measured for a series of 10 independent determinations for 1.45 mg l^{-1} of acetoacetate. The RSD encountered was 1.9%.

One of the main advantages of SPS methods is that sensitivity can be enhanced in proportion to the final volume of the sample to be analysed. The apparent molar absorptivity of our 100-ml sample method, 3.5×10^5 l mol^{-1} cm^{-1}, is higher than the same in solution, 1.6×10^2 l mol^{-1} cm^{-1}. The increase in sensitivity is approximately 2200 times.

The standard deviation of A^*_R, the background absorbance measured for the blank ($A^*_R = A_{590} - A_{720}$), calculated as the average of 10 determinations, is 0.074. The

IUPAC limit of detection was 7.6 $\mu g\ l^{-1}$ for a value of $K = 3$ (confidence level 99.86%) [10].

Effect of foreign ions

The selectivity of the method was tested by using solutions containing 1.43×10^{-2} mM (1.45 $mg\ l^{-1}$) of acetoacetate with various concentrations of different ions and organic species commonly found in human urine. Tolerance is defined as the interfering substance to the acetoacetate ratio (mM/mM) that produces an error of 5% in the determination of the analyte. The results encountered were: urea up to at least a 3500-fold ratio; glucose up to a 1200-fold ratio; lactic acid up to a 310-fold ratio; creatine up to a 95-fold ratio; acetone up to at a 50-fold ratio; cystine, β-hydroxibutyric acid and K^+ up to a 35-fold ratio; NO_3^- up to a 30-fold ratio; Cl^- and uric acid up to a 20-fold ratio; oxalate up to a 2.5-fold ratio; Ca(II) up to a 2-fold ratio; Zn(II) up to a 1-fold ratio and creatinine and SO_4^{2-} up to a 0.5-fold ratio.

The interference was positive for acetone, urea, uric acid and lactic acid, and negative for the other species.

We emphasize that the other ketone bodies and glucose did not show interference in the determination of acetoacetate by this method. The interference level can be reduced by sample dilution. Taking into account the concentration range of the proposed method and the average level of acetoacetate in human urine, a minimum 100-fold dilution is necessary.

Determination of acetoacetate in human urine

The method was applied to the determination of acetoacetate in urine samples of normal and various diabetic subjects by the standard addition method. The major advantage of the proposed method is that no pretreatment of samples is required.

The loss of sensitivity by matrix effect can be evaluated by comparing the slope of the standard calibration graph and the standard-addition calibration graph. The values encountered were 1.60 for normal subjects, and 1.10, 1.02 (previous 1:4 dilution) and 1.03 (previous 1:10 dilution) for the 1, 2 and 3 diabetic specimens, respectively. Therefore, the matrix effect can be eliminated by dilution.

The results obtained for several samples (the average for three determinations) are shown in Table 1. The results are in good agreement with those obtained by the reference methods selected: ^{1}H-NMR and homogeneous nitroprusside methods (Table 1). The nitroprusside method needs strict time control to obtain good results. The ^{1}H-NMR method showed low reproductibility due to several problems: choice of phase and baseline, and integration of signals.

Table 1
Determination of acetoacetate (mM) in human urine

Sample	SPS method	S	Solution method	S	^{1}H-NMR method	S
Normal	0.26	0.03	0.30	0.01	0.26	0.05
Diabetic 1	1.73	0.01	1.67	0.05	—	—
Diabetic 2	3.2	0.12	3.5	0.09	3.2	0.54
Diabetic 3	17.1	0.25	17.0	0.19	18	1.52

The results given are the averages of three determinations.

Table 2
Recovery study of acetoacetate in human urine*

Sample	Amount added (mg l^{-1})	Amount found (mg l^{-1})	Recovery (%)
Normal	—	0.25	—
	0.36	0.60	100
	0.72	1.01	105
Diabetic 1	—	0.44	—
	0.47	0.93	100
	0.93	1.88	102
Diabetic 2	—	0.21	—
	0.47	0.95	102
	0.93	1.85	99
Diabetic 3	—	0.43	—
	0.23	0.65	96
	0.47	0.90	100

*Normal, 1 ml urine; diabetic 1, 0.25 ml urine; diabetic 2, 0.25 ml urine (diluted 1:4); diabetic 3, 0.25 ml urine (diluted 1:10).

To check the accuracy of the proposed method, we carried out a recovery study in the diabetic human urine mentioned above. To do so, we added different amounts of acetoacetate to samples, obtaining acceptable recovery (Table 2).

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[*Received for review 8 August 1988*]