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Journal of Pharmaceutical and Biomedical Analysis
18 (1998) 889–892

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Enzymatic analysis of acetylpolyamine

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Received 15 May 1998; received in revised form 31 July 1998; accepted 16 August 1998

Abstract

An enzymatic method was proposed for measuring acetylpolyamine (AcPA) alone, even when non-acetylated polyamine co-exists. The method consisted of four enzymatic reactions. First, AcPA was hydrolysed by acylpolyamine amidohydrolase to yield acetate; followed by the other three reactions coupled with three enzymes, respectively, acetate kinase, pyruvate kinase, and lactate dehydrogenase; the acetate formation caused a decrease in NADH. The quantity of AcPA was then evaluated as the change in absorbance at 340 nm. The reagent composition of the reaction mixture was determined, and characteristics of the method were investigated. The validation tests produced satisfactory results. The co-existence of non-acetylated polyamine gave no effect on the measurement. The present method was found to be used easily, rapidly and reliably for the selective determination of AcPA itself. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Enzymatic analysis; Acetylpolyamine; Acetylputrescine; Acylpolyamine amidohydrolase; Acetate kinase

1. Introduction

Since Russel et al. [1,2] reported that the quantity of polyamine (PA) in urine of cancer patients was significantly larger than that in normal urine, various methods have been proposed for measuring PA in biological fluids. Most of them were of special instrumental analysis, e.g. the methods belonging to high performance liquid chromatography have been frequently reported. However, they required expensive instruments, and were complicated and tedious. Therefore, they were not suitable for routine clinical chemical analysis, and limited to the basic research use.

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The lack of convenient enzymatic method for measuring PA has hindered advancement in related clinical chemical diagnostics. The situation was complicated by the fact that much of PA in urine exists in acetylated form. Simple enzymatic methods have been reported by Kubota et al. [3] and Matsumoto et al. [4], and the diagnostic kit based on the former has been commercialized. However, they enabled only the measurement of total PA without distinction between acetylated PA, i.e. acetylpolyamine (AcPA), and non-acetylated PA (free PA). Otsuji et al. [5] has reported an enzymatic differential analytical method for PA in urine. This method utilized the difference in substrate specificity between two kinds of

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PII: S0731-7085(98)00226-X

polyamine oxidases for several kinds of free PAs, and distinguished among PAs in their non-acetylated form.

On the other hand, clinically significant PA was reported to be not free PA but AcPA [6–8]. Therefore, a practical enzymatic analytical method, distinguishing AcPA from free PA, was desired to be developed, but there have been no papers approaching that.

In this paper, an enzymatic method for measuring AcPA alone, even when free PA co-exists, has been proposed. This method is rapid and convenient, and does not require any special or expensive instruments.

2. Experimental

2.1. Materials

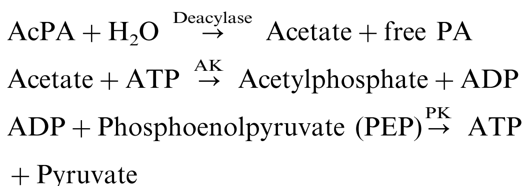
Acetylpolyamine amidohydrolase (deacylase, from *Streptomyces avellaneus*) was kindly supplied by A & T Ltd., Tokyo, Japan. Acetate kinase (AK, EC 2.7.2.1, from *Bacillus stearothermophilus*) was kindly supplied by Unitika Ltd., Osaka, Japan. Pyruvate kinase (PK, EC 2.7.1.40, from rabbit muscle), lactate dehydrogenase (LDH, EC 1.1.1.27, from hog muscle), ATP and NADH were obtained from Boehringer Mannheim GmbH, Mannheim, Germany. Acetylputrescine (AcPut) was obtained from Sigma Chemical Co., St. Louis, MO. The other chemicals were commercial products of analytical grade.

2.2. Instrumentation

The absorbance at 340 nm was measured with the Shimadzu UV 2200 spectrophotometer.

2.3. Principle

The analytical principle is as follows:



$$\text{AcPA} + \text{H}_2\text{O} \xrightarrow{\text{Deacylase}} \text{Acetate} + \text{free PA}$$

$$\text{Acetate} + \text{ATP} \xrightarrow{\text{AK}} \text{Acetylphosphate} + \text{ADP}$$

$$\text{ADP} + \text{Phosphoenolpyruvate (PEP)} \xrightarrow{\text{PK}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

The above schematic principle is consisted of four enzymatic reactions: First, AcPA is hydrolyzed by deacylase to yield acetate, and finally, the acetate formation causes a decrease in NADH. The quantity of AcPA is then evaluated as the change in absorbance at 340 nm.

2.4. Procedure

AcPut was used as a typical AcPA in urine. A 0.9 ml vol of the reaction mixture was pre-incubated at 30°C for 5 min, and 0.1 ml of AcPut aq solution was added as a sample. The composition of the reaction mixture was as follows (final concentration): Tris–HCl buffer (pH 7.8, 0.1 M); 3.5 mM ATP; 0.7 mM PEP; 0.4 mM NADH; 8.0 mM $MgSO_4$; 60.0 mM KCl; 20.0 U ml^{-1} AK; 9.0 U ml^{-1} PK; 3.0 U ml^{-1} LDH; 8.0 U ml^{-1} deacylase. Each concentration including pH was determined in a preliminary experiment, considering the concentration range not having an influence on the measurement. After addition of AcPut, the change in absorbance at 340 nm was measured within 10 min.

Table 1
Method validation

Linearity (n = 11; 50–100 μM)
Slope ± SD = 1.002 ± 0.004; RSD = 0.40
Intercept ± SD = 1.273 ± 2.397
r = 0.9998
Limit of detection (LOD) and limit of quantification (LOQ)
LOD = 13.7 μM; LOQ = 42.7 μM
Precision (100, 300, 500 μM; n = 8, k = 5)
100 μM Repeatability (RSD): 1.27%
Reproducibility (RSD): 1.40%
300 μM Repeatability (RSD): 1.11%
Reproducibility (RSD): 1.22%
500 μM Repeatability (RSD): 1.23%
Reproducibility (RSD): 1.34%
Accuracy, analytical recovery (100, 300, 500 μM; n = 5) (95% confidence limits)
102.8 ± 3.45 μM (102.8 ± 3.5%)
297.2 ± 6.56 μM (99.1 ± 2.2%)
507.0 ± 10.04 μM (101.4 ± 2.0%)

3. Results

3.1. Linearity

A sample with a high AcPut concentration (1.0 mM) was diluted in the concentration range 50 μM–1.0 mM. A linear relationship existed over a full range up to 1.0 mM with a high correlation coefficient (r) of 0.9998 (Table 1). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the intercept of the regression line, assuming that the former is equal to three times the standard deviation (SD) of the blanks and the latter ten times blank SD. LOD and LOQ were 13.7 and 42.7 μM, respectively, which reached a practical level.

3.2. Precision

The results of within-day repeatability and between-day reproducibility tests using samples with three AcPut concentrations are shown in Table 1. The former test was carried out eight times within one day, and the latter five consecutive days. Precision parameters were satisfactory for clinical chemical analysis, and very little different from each other.

3.3. Analytical recovery

AcPut solutions were added to normal human urine at 100, 300 and 500 μM concentrations. As shown in Table 1, the method was found to be sufficiently accurate. The biological fluid did not affect the measurement recognizably. The comparison between within-run and between-run assays did not make a statistical difference: $F\text{obs} = 1.95 < F(0.05, 2, 12) = 3.89$.

3.4. Effect of co-existents

An interference study was performed involving the addition of the following substances that may co-exist in urine: Uricate (0–20 g l^{-1}), ascorbate (0–100 g l^{-1}), lactate (0–15 g l^{-1}) and glutathione (0–30 g l^{-1}). No significant interference was observed.

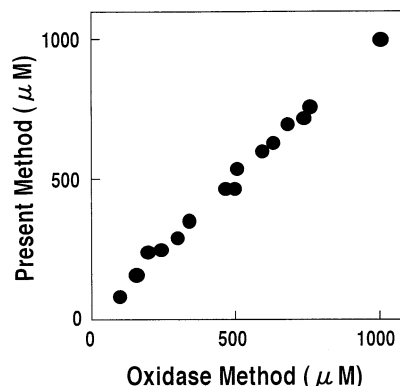


Fig. 1. Correlation between concentrations of acetyl putrescine (AcPut), determined by the present method and Oxidase Method. A kit under the trade name Polyamine Test Enzyme (A & T Ltd., Tokyo, Japan) was used as Oxidase Method. Since Oxidase Method was not able to be applied to the determination of AcPut in a sample including non-acetylated polyamine, aq solution of AcPut alone was used as the sample (see Text).

3.5. Effect of non-acetylated putrescine

Non-acetylated putrescine (free Put, 0–1.0 mM) was added to samples. Any effects were not observed.

3.6. Correlation with the enzymatic method for the determination of total polyamine

The enzymatic diagnostic kit for measuring total polyamine in urine, mentioned in Section 1, were based on the coupling enzyme reactions of two enzymes, i.e. deacylase and putrescine oxidase. This kit (Oxidase Method) are now commercially available, but AcPut can not be determined selectively by this kit. Therefore, the present method was compared with Oxidase Method in the absence of free PA in a sample. For 15 samples with various concentrations of AcPut only and without free PA, the regression equation was favorably expressed as follows: $y = 0.99x + 0.96$ ($r = 0.996$) (Fig. 1).

4. Discussion

A new enzymatic method was developed for the

determination of AcPut, as a typical AcPA in urine, with deacylase and AK as key enzymes which are both commercially available. AK has high substrate specificity for acetate, and was observed to be useful for measuring choline esterase activity in biological fluids [9]. The biological fluid also did not interfere with the present measurements. Deacylase has been used in Oxidase Method for measuring total PA. It has high V_{max} values for AcPut and acetylcadaverine, and lower K_m value for AcPut [10]. In addition to AcPut, acetylcadaverine will probably be determined by the present method. However, considering its higher K_m value and lower concentration in urine, this method may have somewhat high selectivity in AcPut. A variety of AcPAs should also be able to be determined by the present method if deacylases with different substrate specificities become to be available. For example, Fujishiro et al. [11] isolated acetylpolyamine amidohydrolase from *Mycoplana bullata* cell. This enzyme showed broad substrate specificity for several AcPAs, such as AcPut, acetylcadaverine, acetylspermidine and acetylspermine, which suggests the possibility of the equal measurement of these AcPAs. However, this enzyme has not been commercially available.

The present method was based on measuring the absorbance of NADH at 340 nm, which made us expect high reliability of the measurement. The reaction proceeded stoichiometrically, and a dilution test showed a good linearity over a wide range. A good precision and accuracy were observed in repeatability, reproducibility and recovery tests. None of co-existents including reductants, which are apt to interfere with oxidase reaction, influenced the determination of AcPut, and especially, the co-existence of free Put had no influence, which shows the inertia to reductants and the high selectivity or specificity of the present method for acetylated PA.

The present method was the first one for measuring the quantity of AcPA itself, and produced a satisfactory result. It was also noteworthy that the method was found to be used easily, rapidly and simply for the determination of AcPA. Clinical application remains to be investigated.

Acknowledgements

The authors are grateful to A & T Ltd. for the supply of deacylase and a diagnostic kit, Polyamine Test Enzyme, and Unitika Ltd. for the supply of AK. The authors are also grateful to Dr M. Okada of Tokuyama Ltd. for his advice about the properties of deacylase and Mr. T. Hosokawa of Kyowa Hakko Kogyo Ltd. for his advice about the validation process.

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