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Note

Biochemical indices of renal damage: separation of urinary alanine aminopeptidase by liquid chromatography

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Alanine aminopeptidase (AAP) (EC 3.4.11.2) is a membrane-bound enzyme which is different from classical leucine aminopeptidase [1]. It has been isolated from several human tissues [2,3] and body fluids [4].

Increased serum AAP activity was detected in various disorders, especially in hepatobiliary diseases. Low activity is detectable in the urine of healthy individuals, but it is greatly increased after primary or secondary renal damage resulting from renal failure, transplant rejection, nephrotoxicity, hypertension or diabetes [5–7]. Urinary AAP activity is an especially sensitive indicator of early kidney injury: it increases before other renal function test results become abnormal [8]. In spite of its clinical significance, AAP is not commonly measured in the routine clinical laboratory, mainly because of the lack of suitable methods for rapid analysis. Its low activity in urine creates a requirement for relatively long incubation periods. In addition, urine contains inhibitor [9] which must be diluted or removed before assay. So far, only spectrophotometric or radioimmunoassay methods have been reported for AAP analysis. In this paper we describe the development and validation of a high-performance liquid chromatographic (HPLC) method for the separation of AAP. We also report an increased urine concentration of AAP in patients with chronic renal failure.

EXPERIMENTAL

Reagents

All reagents were of HPLC grade, including acetonitrile from Carlo Erba (Milan, Italy) and water from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Trifluoroacetic acid was purchased from Sigma (St. Louis, MO, U.S.A.).

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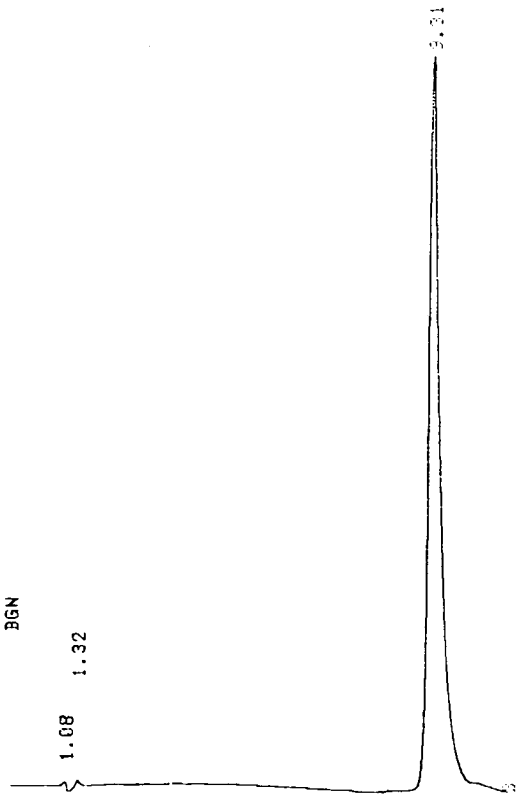

Fig. 1. Chromatogram of AAP reference compound. The retention time is indicated. Column: reversed-phase HS-5 C_{18}. Elution with linear gradient from 50 to 80% acetonitrile in water containing 0.1% trifluoroacetic acid. Flow-rate, 1.5 ml/min. Detection, UV (220 nm).

Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were used to purify the sample before chromatography.

Purified AAP reference compound was obtained from human kidney according the method of Hiwada et al. [10]. L-Alanyl-β-naphthylamide was obtained from Sigma.

Urine samples

Normal urine samples were collected from the personnel of our institute. All had normal renal function and their ages ranged from 23 to 50 years with a mean of 44.8 years.

Pathological samples were obtained from ten patients with chronic renal failure (glomerulonephritis), with ages ranging from 26 to 55 years with a mean of 42.5 years.

Each specimen from normal subjects and patients was collected from 7:00 to 10:00 a.m. to prevent any influence of circadian variation and aliquots were immediately subjected to purification with Sep-Pak C_{18} cartridges to remove the interfering substances, then successively analysed by HPLC.

TABLE I

RECOVERY OF AAP ADDED TO URINE

Amount added (μg/ml)	Amount recovered ($n=4$) (μg/ml)	Recovery (%)
1	0.83	83.0
10	7.95	79.5
100	74.50	74.5
Mean ± S.D.		79 ± 4.27

Sample purification

The cartridge was washed with methanol and water as recommended by the manufacturer, then equilibrated with 5 ml of 15% acetonitrile in water. After 10 min, 1 ml of sample was loaded on to the cartridge, which was washed with 3 ml of 15% acetonitrile in water. The eluate was discarded. The AAP was then eluted with 1 ml of 60% (v/v) acetonitrile in water, collected and evaporated. The dried extract was reconstituted in 100 μl of 15% acetonitrile in water and a 20-μl aliquot was analysed by HPLC.

High-performance liquid chromatography

The apparatus used was a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 3B equipped with dual pumps capable of generating a gradient elution and an HS-5 C_{18} column (12.5 × 0.4 cm I.D.) (Perkin-Elmer). Chromatograms were recorded by monitoring the absorbance at 220 nm using an LC 85 UV detector (Perkin-Elmer) and the data were processed with LCI-100 integrator (Perkin-Elmer). Samples were analysed by gradient elution at a flow-rate of 1.5 ml/min with a 20-min linear gradient from 50 to 80% acetonitrile containing 0.1% trifluoroacetic acid.

Assay of enzyme activity

The activity of AAP was assayed by the method of Jung and Scholz [11] using L-alanyl-β-naphthylamide as substrate. The statistical analysis was performed by Student's *t*-test.

RESULTS

Fig. 1 shows the separation of AAP reference compound by HPLC. The calibration graph indicates that the method is linear between 0.2 and 10 μg ($r=0.99$; $y=0.6+3.8x$). The detection limit at a signal-to-noise ratio higher than 2 was 0.2 μg. The results of the purification of urine samples with Sep-Pak C_{18} cartridges are presented in Table I. The analytical recovery (79%) was determined by measuring urine samples spiked with three different levels of AAP reference compound ($n=4$).

The representative chromatograms of AAP from the controls and patients are illustrated in Fig. 2. The AAP peak was identified by comparison of its retention

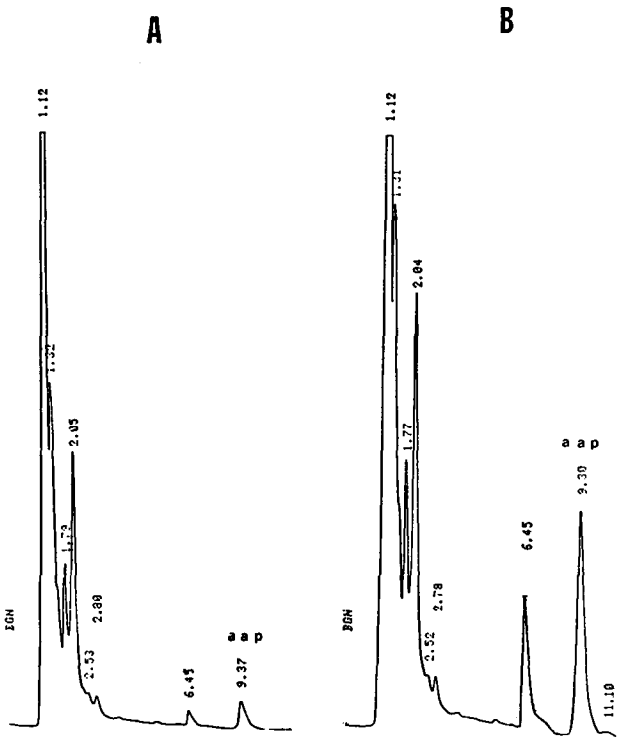

Fig. 2. Chromatograms of AAP in urine from (A) a control and (B) a patient. The retention time is indicated. Experimental conditions as in Fig. 1.

time with that of the reference compound, co-chromatography with the standard and absorbance ratios.

The results of the determination of the urinary AAP concentration in ten controls and ten patients with chronic renal failure are presented in Fig. 3. The mean $\pm$ S.D. values were 0.4 ± 0.06 μg/ml and 2.06 ± 0.5 μg/ml, respectively. The patients had significantly higher urinary AAP aminopeptidase levels ($p < 0.001$) than the controls.

Fig. 4 shows the correlation between the urinary AAP concentration determined by HPLC and the AAP activity determined by the method of Jung and Scholz [11].

DISCUSSION

The kidney has considerable functional reserve capacities. Thus routine diagnostic tests for kidney disease based on renal function inherently lack the sensitivity required for early detection. A different approach is the evaluation of renal cellular integrity by measuring enzyme activities released predominantly from tubular cells into the urine. The utility of the enzyme assays in urine has been demonstrated in clinical and experimental toxicology [12], screening for renal disease [13] and early warning of rejection in kidney transplant patients. Over

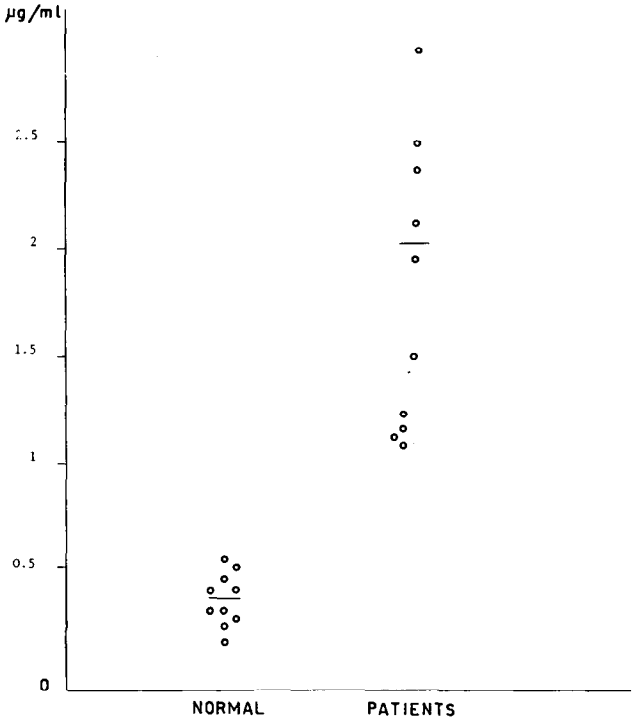

Fig. 3. AAP concentrations in ten controls and ten patients.

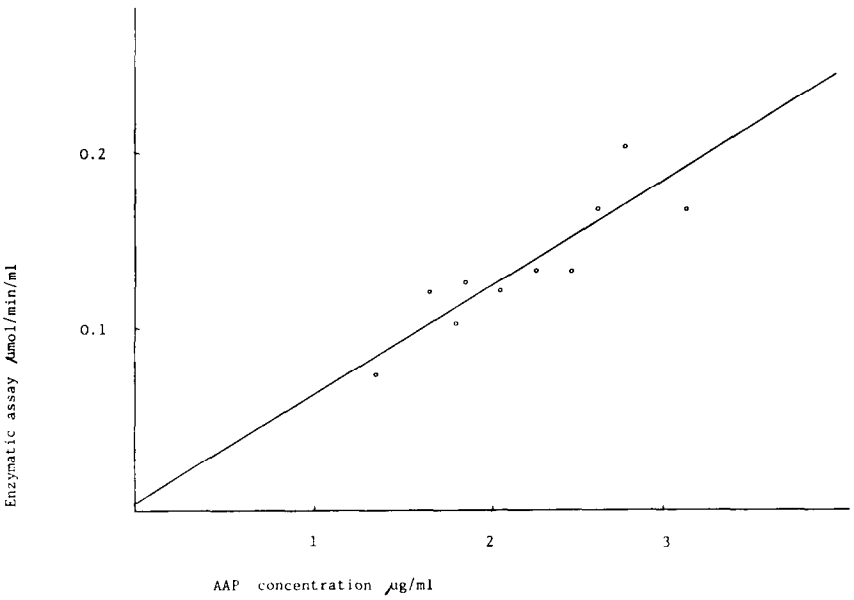

Fig. 4. Correlation between AAP concentration and activity using L-alanyl-β-naphthylamide. $r=0.77$

thirty enzymes of renal origin have been identified in urine. Of these, AAP is an especially sensitive indicator: its activity increases before other renal function test results become abnormal. Even though AAP can be measured in different ways, most clinical studies have been performed by measuring the enzyme activity. With this measure, however, the reported normal values from serum and urine differ markedly owing to the various ways of treating the samples and the presence of interfering substances. The separation by HPLC of AAP and the measurement of its concentration rather than its activity can be expected to provide more accurate information about the true levels in biological samples.

The concentrations of urinary AAP determined in the control group varied from 0 to 0.5 μg/ml. Owing to the sensitivity of our method, even minute increases are readily revealed. Significantly increased concentrations were found in patients with chronic renal failure, which suggests that AAP might be a suitable marker of renal tubular damage. The HPLC procedure described is simple, reproducible and offers a useful alternative to other methodologies currently in use.

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