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# Aspartate aminotransferase isoenzymes — differential kinetic assay in serum

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## Summary

The isoenzymes of aspartate transaminase differ in their kinetic properties in that the cytoplasmic isoenzyme is more readily inhibited by adipate and by 2-oxoglutarate (substrate) at low pH. A differential kinetic assay based on this phenomenon has been optimised for use in assays of serum samples. The new method agrees well with an immune absorption procedure. Methods based on chromatographic separation of the isoenzymes fail in the presence of serum.

## Introduction

The two isoenzymes of aspartate aminotransferase (AAT, EC 2.6.1.1) are normally located in the cytoplasm (cAAT) and mitochondria (mAAT) respectively. Both are present in serum (or plasma) so that they are assayed together when the total AAT activity of serum is measured. It may be of value clinically to measure the activity of each independently, but there are objections to many of the methods which have been proposed for this purpose (see 'Discussion'). It seemed worthwhile, therefore, to re-examine the differential kinetic assay, introduced by Fleisher et al. [1], with a view to optimising its performance as a discriminant between the isoenzymes in the presence of the analytical matrix (serum). This is particularly important in that many other possible methods are not appropriate for clinical use.

## Methods and results

### *AAT isoenzyme assay*

Our finally chosen method requires two separate measurements of rate of change of absorbance (340 nm) at 37°C with the following final reagent concentrations and pH values:

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pH 7.4: potassium/phosphate buffer 33 mmol/l, L-aspartate 33 mmol/l, 2-oxoglutarate 6.7 mmol/l, malate dehydrogenase (MDH) 833 U/l (1 U per 1.2 ml test volume), NADH 0.125 mmol/l (0.1 mg per 1.2 ml test volume), serum sample/total volume ratio 1 : 12 (0.1 ml sample per 1.2 ml test volume).

pH 6.0: potassium/phosphate buffer 10 mmol/l, adipate 50 mmol/l, L-aspartate 15 mmol/l, 2-oxoglutarate 15 mmol/l, MDH, NADH and sample volume/total volume ratio as above.

In measurements at pH 6.0 it is essential that the sample be preincubated with 2-oxoglutarate for several min at 37°C before aspartate is added to start the AAT reaction [2].

Once the two rate measurements have been obtained on a mixture such as serum, containing both isoenzymes, the two separate activities may be calculated, provided that it is known how the activity of each isoenzyme individually varies between the two sets of conditions employed. This information is expressed, for each isoenzyme, as the quotient, activity at pH 6.0 divided by activity at pH 7.4. For these quotients we employ the abbreviations $M$ for mAAT and $C$ for cAAT. The values finally adopted for clinical use are $M = 0.90$, $C = 0.05$. $M$ and $C$ are dimensionless ratios.

The calculation may be simplified as follows for a 1-cm light path and conditions as described above:

$$\text{cAAT activity (U/l)} = 2270 \times (0.9\ V_1 - V_2)$$

$$\text{mAAT activity (U/l)} = 2270 \times (V_2 - 0.05\ V_1)$$

where $V_1$ = rate of change of absorbance at pH 7.4, $V_2$ = rate of change of absorbance at pH 6.0, and both activities are finally expressed in terms of activity measured under the above set of pH 7.4 assay conditions.

*Purification of isoenzymes*

The cAAT and mAAT used in this work were purified from human liver and heart, obtained at autopsy from organs judged to be free of disease, by a method modified from that of Glatthaar et al [3]. Typical specific activities achieved were cAAT 121 U/mg, mAAT 267 U/mg protein.

*Optimisation*

The essential content of this work was to discover assay conditions under which $M$ and $C$ were as distinct as possible and yet were also insensitive to chance variations in those assay conditions, not forgetting the sample itself as a source of variation. After preliminary experiments with purified isoenzymes alone we proceeded to add serum (correcting measured rates for the activity already present in the individual serum sample) and determined $M$ and $C$ over a wide range of conditions; varying one component of the assay mixture at a time, including the nature and concentration of buffer ions. When approximately optimal conditions had been identified, the cycle of variation of conditions was again repeated more than once. Figs. 1 and 2 illustrate stages close to the last step in this process.

It will be seen that the substrate concentrations finally chosen do not give the highest possible value of the ratio $M/C$. Similarly, if adipate concentrations were increased beyond 50 mmol/l, higher values were observed for $M/C$. The magnitude

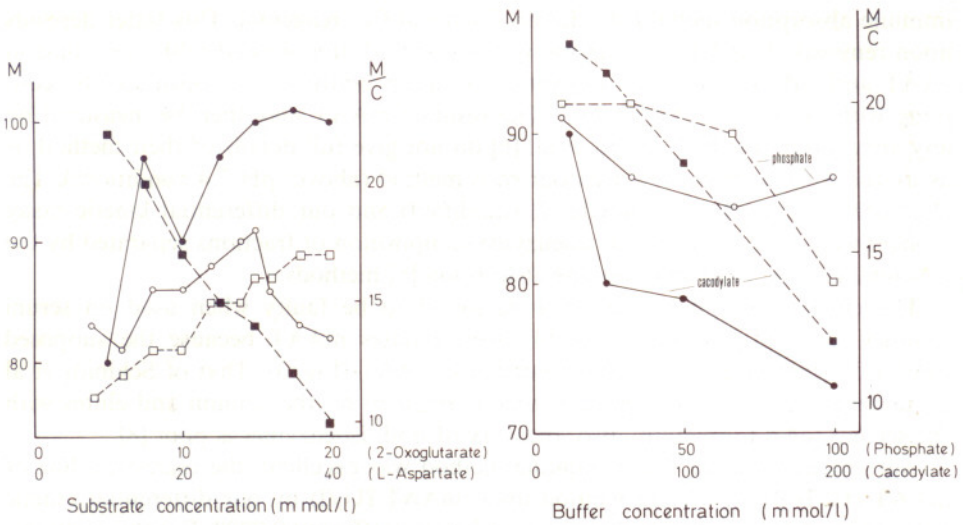


Fig. 1. Variation of activity quotients with substrate concentration. For definitions of $M$ and $C$, see text. In the pH 6 measurements the buffer was 33 mmol/l phosphate, adipate concentration was 50 mmol/l, and substrate concentrations were varied. Conditions for measurements of rate were in other respects as described in the text. The samples used were mixtures of human serum with purified isoenzyme and results are corrected for pre-existing activity in the serum. ○, □, $M$ and $M/C$ respectively with L-aspartate held constant at 30 mmol/l and 2-oxoglutarate varied as shown; ●, ■, $M$ and $M/C$ respectively with 2-oxoglutarate held constant at 15 mmol/l whilst L-aspartate was varied as shown.

Fig. 2. Variation of activity quotients with buffer concentration. In the pH 6 measurements L-aspartate concentration was 15 mmol/l, 2-oxoglutarate 15 mmol/l, adipate 50 mmol/l, and the nature and concentration of buffer was varied as shown. Conditions were otherwise as described in the text. ○, □, $M$ and $M/C$ respectively with phosphate buffer; ●, ■, $M$ and $M/C$ respectively with cacodylate buffer.

of this ratio, however, is not the sole criterion for choice of assay conditions (see 'Discussion'). Under conditions where $M$ was considerably less than 0.90, or $C$ more than 0.05, variability in the measurement of these quotients increased to an undesirable extent.

In further experiments, different individual serum samples were used as matrix and it was found that measured values of $M$ and $C$ varied considerably — more than can be attributed to error in the measurements themselves. For example, in one series with isoenzymes from human heart added to different serum samples we found for cAAT (49 samples, mean $\pm$SD) $C = 0.057 \pm 0.019$, for mAAT (47 samples, mean $\pm$SD) $M = 0.904 \pm 0.196$. These were single determinations on each serum sample and are intrinsically of high variability because activity due to added isoenzyme at each pH value must be calculated from the difference between activity with added isoenzyme and that determined for serum alone.

*Validation, normal values*

Detailed comparative studies were made with two chromatographic [4,5], and one

immune absorption method [6] (Eiken Chemical Co. reagents). This latter depends upon removal of cAAT from serum by reaction with freeze-dried rabbit anti-human cAAT antibody followed by adsorption to sheep erythrocytes 'sensitised' by coupling with antibody to rabbit IgG. The residual mAAT may then be measured by any appropriate method. Wada et al. [6] do not give full details of their method of assay for AAT activity. We used our own method (above; pH 7.4 conditions). The electrophoretic method of Boyde [7] (modified) and our differential kinetic assay (above) were used to check the isoenzyme composition of fractions separated by the chromatographic [4,5] and immune absorption [6] methods.

The chromatographic methods were found to be faulty when used on serum samples. That of Sampson et al [4] overestimates mAAT because the supposed mAAT fraction is heavily contaminated with cAAT (Fig. 3). That of Schmidt et al [5] underestimates mAAT because some is retained on the column and elutes with the second (cAAT) fraction: also, recovery of both isoenzymes is poor [8].

Agreement with the Wada immune method was excellent, the regression line of mAAT (U/l) by the Wada method upon mAAT (U/l) by our differential kinetic assay being $0.501 + 1.004x$; Pearson correlation coefficient 0.9998. For this comparison, 102 pairs of determinations were carried out on serum samples with mAAT varying from 1.4 to 955 U/l. No cAAT could be detected in the supernatants following adsorption to sheep erythrocytes, though an earlier version of the assay from the same manufacturer was less successful in this respect.

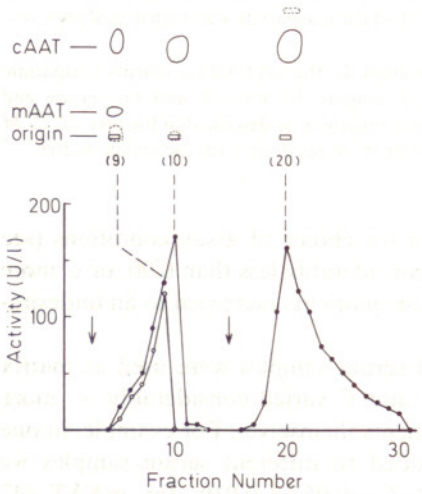


Fig. 3. Differential analysis of chromatographic fractions obtained by the method of Sampson et al. [4]. A natural (pathological) serum sample containing 1330 U/l AAT activity was subjected to chromatography as described in [4] and each fraction assayed by our differential kinetic assay method. Arrows show points of application of the serum sample and of the second eluting buffer. Results are presented as the activity actually found in each fraction (U/l), ○, mAAT activity, ●, total AAT activity. Above the graphs are tracings of AAT activity patterns found after electrophoresis [7] of fractions 9, 10 and 20. The mAAT band from fraction 9 is characteristically compact owing to $\alpha_2$-macroglobulin binding. No cAAT should be detected in fractions 9 and 10 if the chromatographic method is functioning correctly, but as shown here it typically was present.

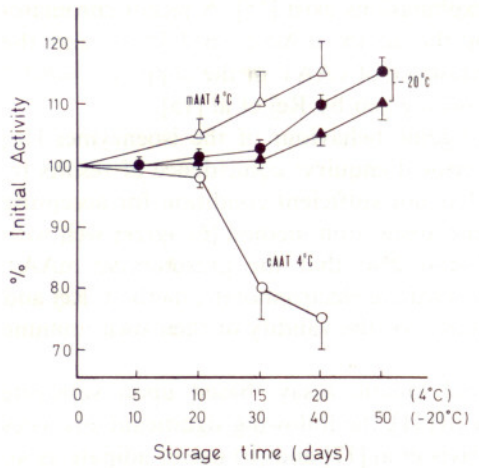


Fig. 4. Changes in measured activity of isoenzymes on storage; % of initial activity, mean ± SD. Natural serum samples were stored at 4°C or −20°C and the activity of mAAT and cAAT measured at intervals by the method described in text. For the −20°C study the samples were frozen in small aliquots, thawed once only for assay and not re-frozen. △, ○, mAAT and cAAT respectively, 47 samples stored at 4°C, initial mAAT 5.5–13.5 U/l, initial cAAT 20–45 U/l. ▲, ●, mAAT and cAAT respectively, 17 samples stored at −20°C, initial mAAT 10–25 U/l, initial cAAT 40–65 U/l.

Recovery was tested on the final form of the assay procedure by adding purified isoenzymes to serum samples. For mAAT we found 96.5 ± 3.5%, for cAAT 103 ± 4.0% (mean ± SD, 20 determinations).

Reproducibility was tested within and between batches by 10 or more assays on natural clinical samples at various activity levels. Results for inter-batch variation were as follows (mAAT activity U/l, mean ± SD): 4.3 ± 0.52, 18.5 ± 1.5, 47.0 ± 2.5, 668 ± 24.

Upon storage at 4°C the measured activity of serum mAAT rose progressively whilst that of cAAT fell: on storage at −20°C, both activities rose progressively (Fig. 4). It is suggested that storage be limited to 7 days at 4°C or 30 days at −20°C.

Normal values of serum mAAT by our kinetic differential assay method are as follows (U/l at 37°C and under our pH 7.4 assay conditions: mean ± SD); no age correlation was detected: males (116 men aged 18–81): 4.94 ± 1.05 (cAAT 17.4 ± 4.15); females (114 women aged 16–82): 4.43 ± 1.02 (cAAT 16.85 ± 4.85).

## Discussion

Separation of cAAT and mAAT looks easy but is not, especially in the presence of serum. $\alpha_2$-Macroglobulin binds mAAT in such a way as to disturb its behaviour on DEAE cellulose chromatography [9], electrophoresis [7,10] and ultracentrifugation [11]. Failure to detect mAAT in normal serum by certain electrophoretic and chromatographic methods (e.g. [12–14]) is probably attributable, at least in part, to

this phenomenon, though other possible explanations exist [15]. A recent chromatographic method [4] gives high values for the serum mAAT level [8,15] and the discrepancy has now been traced to the presence of cAAT in the supposed mAAT fractions. Less direct evidence of this has been given by Rej et al [15].

Methods depending on the distinct antigenic behaviour of the isoenzymes [16] should also be investigated in a spirit of critical enquiry: coincidence of results by two independent methods is a necessary but not sufficient condition for accepting both as sound. As shown above, an immune absorption method [6] agrees well with our differential kinetic assay and we found also that the presumptive mAAT fractions contain no cAAT detectable by a sensitive electrophoretic method. Rej and coworkers [15] have presented strong evidence for the validity of their own immune precipitation method.

Since the introduction of a differential kinetic assay, based upon substrate (2-oxoglutarate) inhibition of cAAT at low pH [1], the following significant advances have been made: (a) the error theory of Davis et al [17] and (b) use of adipate as an additional inhibitor of cAAT [18]. Apparently, neither of these groups, nor that of Graubaum et al [19], conducted optimisation studies in the presence of serum, a step which we have found to be essential, and it has been shown that the Graubaum method overestimates mAAT [15]. Martinez-Carrion's group [18] was not concerned with serum assay.

We found many excellent combinations of conditions giving very clear discrimination between mAAT and cAAT in mixtures of the purified isoenzymes. But with the exception of conditions close to those finally adopted, the values of $M$ and $C$ altered drastically and for the worse as soon as serum was added to test for matrix effects.

Davis et al [17] indicated that the search for ideal conditions should be directed to achieve a high $M/C$ ratio whilst at the same time preserving a high value for $M$; that is, not overemphasising the minimisation of $C$; and our experience confirms that this is sound. They assumed, however, that the values of $M$ and $C$ could be determined with whatever degree of precision may be required by repeated measurements, whereas we have found that precision is restricted in practice by variation in matrix effect between one serum sample and another.

We accordingly decided to pick reasonable but, in the last analysis, arbitrary values of $M$ and $C$. It can easily be shown and has been determined in practice that small variations or errors in $M$ and $C$, in the vicinity of $M = 0.90$ and $C = 0.05$, have little effect on the calculated activities of mAAT and cAAT ([8], p. 144). Thus, although there is an irreducible limit to the accuracy of measurements by our proposed method, we are confident that it is satisfactory for clinical purposes. It is, moreover, simple, cheap, quick and robust.

A few technical points require comment:

Larger amounts of MDH may cause trouble because of trace amounts of other enzymes present as impurities in commercially-available preparations.

We have experienced no problem with instability of NADH at pH 6.0. Reagent blanks, which should be run at each pH value with each batch of determinations, average 0.0005 per min at each pH value, which is exactly as would be calculated for

our reaction conditions [20]. The very high reagent blanks, at pH 6, found by Rej et al [15], cannot be easily explained (but see [2]).

We have experienced no problem, either, with the pyruvate and lactate dehydrogenase invariably present in serum, presumably because of the preincubation period of at least 8 min used as routine at both pH values.

It may be argued that we should have used the IFCC Standard Method instead of our pH 7.4 assay conditions. The chief reason why this was not done is that the conditions of the IFCC method, having been developed in respect of samples containing substantially only one of the isoenzymes (cAAT), are seriously suboptimal for mAAT, which requires approximately equal concentrations of L-aspartate and 2-oxoglutarate for maximum activity [9]. Our pH 7.4 conditions on the other hand, derived from the original UV kinetic method of Karmen [21] and perhaps still the most widely used in clinical laboratories all over the world, are about equally suboptimal for the two isoenzymes ([9] and unpublished results). Further, the use of pyridoxal phosphate in assay mixtures leads to the apoenzyme being assayed together with holoenzyme, whereas it is a different substance worthy of independent study, as has already appeared from clinical surveys [22–24].

**Acknowledgements**

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**References**

1 Fleisher GA, Potter CS, Wakim KG. Separation of two glutamic-oxalacetic transaminases by paper electrophoresis. Proc Soc Exp Biol Med 1960; 103: 229–231.

2 Boyde TRC. A nonenzymic reaction that interferes with measurement of serum aspartate aminotransferase activity at pH 6. Clin Chem 1981; 27: 1783–1784.

3 Glatthaar BE, Barbarash GR, Noyes BE, Banaszak LJ, Bradshaw RA. The preparation of the cytoplasmic and mitochondrial forms of malate dehydrogenase and aspartate transaminase by a single procedure. Anal Biochem 1974; 57: 432–451.

4 Sampson EJ, Miller SA, McKneally SS, Whitner VS, Hannon WH, Burtis CA. Column chromatographic separation of isoenzymes of aspartate aminotransferase. Clin Chem 1978; 24: 1805–1812.

5 Schmidt E, Schmidt FW, Mohr J. An improved simple chromatographic procedure for separating the isoenzymes of malic dehydrogenase and glutamic oxalacetic transaminase. Clin Chim Acta 1967; 15: 337–342.

6 Wada H, Teranishi H, Kagamiyama H et al. A simple immunological method for differential determination of serum glutamic-oxalacetic transaminase isoenzymes. I. Using anti-human-GOT antibody. Med J Osaka Univ 1978; 29: 181–190.

7 Boyde TRC. Detection and assay of mitochondrial aspartate aminotransferase in serum. Z Klin Chem Klin Biochem 1968; 6: 431–435.

8 Kwong EML. Development of methods for the routine assay of mitochondrial aspartate aminotransferase in serum, and applications in the study of human disease. PhD Thesis, University of Hong Kong, 1980.

9 Boyde TRC. Aspartate aminotransferase isoenzymes. Fundamental and clinical studies. MD Thesis, University of London, 1967.

10 Boyde TRC. The interaction between $\alpha_2$-macroglobulin and cationic aspartate aminotransferase. Biochem J 1969; 111: 59–61.
11 Boyde TRC, Pryme IF. $\alpha_2$-Macroglobulin binding of trypsin, chymotrypsin, papain, and cationic aspartate aminotransferase. Clin Chim Acta 1968; 21: 9–14.
12 Murros J, Konttinen A, Somer H. An electrophoretic method for the quantitation of aspartate aminotransferase isoenzymes. Clin Chim Acta 1972; 41: 263–268.
13 Katsunuma T, Kamiguchi H, Sase M. Separative assay method for aspartate transaminase using plate electrophoresis and diazonium salts. Tokai J Exp Clin Med 1976; 1: 235–239.
14 Schmidt E, Schmidt FW, Otto P. Isoenzymes of malic dehydrogenase, glutamic oxalacetic transaminase and lactic dehydrogenase in serum in diseases of the liver. Clin Chim Acta 1967; 15: 283–289.
15 Rej R, Bretaudiere J-P, Graffunder B. Measurement of aspartate aminotransferase isoenzymes: six procedures compared. Clin Chem 1981; 27: 535–542.
16 Nisselbaum JS, Bodansky O. Immunochemical and kinetic properties of anionic and cationic glutamicoxalacetic transaminases separated from human heart and human liver. J Biol Chem 1964; 239: 4232–4236.
17 Davis JE, Hicks GP, Sherry AE. Minimizing errors in differential kinetic analysis of isoenzymes. Determination of aspartate aminotransferase (GOT) using differential substrate levels. Anal Biochem 1972; 48: 569–580.
18 Martinez-Carrion M, Barber B, Pazoles P. On the determination of isozyme levels in preparations containing cytoplasmic and mitochondrial aspartate aminotransferase. Biochim Biophys Acta 1977; 482: 323–329.
19 Graubaum HJ, Ehrenberg K, Wagenknecht C. Eine einfache kinetische Methode zum Nachweis von zytoplasmatischer und mitochondrialer GOT ohne vorherige Auftrennung. Acta Biol Med Germ 1974; 32: 293–297.
20 Lowry OH, Passonneau JV, Rock MK. The stability of pyridine nucleotides. J Biol Chem 1961; 236: 2756–2759.
21 Karmen A. A note on the spectrophotometric assay of glutamic-oxalacetic transaminase in human blood serum. J Clin Invest 1955; 34: 131–133.
22 Rosalki SB, Bayoumi RA. Activation by pyridoxal 5-phosphate of aspartate transaminase in serum of patients with heart and liver disease. Clin Chim Acta 1975; 59: 357–360.
23 Moss DW. Reactivation of the apoenzyme of aspartate aminotransferase in serum. Clin Chim Acta 1976; 67: 169–174.
24 Ratnaike, S, Moss DW. Apoenzyme of aspartate aminotransferase in serum in health and disease. Clin Chim Acta 1977; 74: 281–288.