Journal of Chromatography, 495 (1989) 105–112
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4903

DETERMINATION OF SERUM CYTIDINE DEAMINASE ACTIVITY USING ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY

IAN T JAMES

The Bone and Joint Research Unit, London Hospital Medical College, 25-29 Ashfield St Whitechapel, London E1 (U K)

KARL HERBERT and DAVID PERRETT*

The Dunn Laboratories, St Bartholomew's Hospital, West Smithfield, London EC1 7BE (U K)

and

PAUL W THOMPSON

Department of Rheumatology, The London Hospital, Whitechapel, London E1 (U K)

(First received February 21st, 1989, revised manuscript received June 8th, 1989)

SUMMARY

A rapid and sensitive assay for serum cytidine deaminase has been developed utilising ion-pair reversed-phase high-performance liquid chromatography The addition of 1-octanesulphonic acid (OSA) caused the retention of cytidine and uridine to reverse and uridine, the minor component in the assay, to elute first Cytidine, uridine and allopurinol (internal standard) were separated on a 5-μm Hypersil ODS column using 100 mM ammonium acetate with 1% (v/v) methanol and 1 mM OSA adjusted to pH 5 0 Detection was at 262 nm Peak areas were linear from 7 pmol to 6 nmol injected ($r=0\ 99$) Intra-assay variation was 7 8% ($n=10$) and the correlation with a colorimetric assay was $r=0\ 78$ ($p<0\ 001$)

INTRODUCTION

Cytidine deaminase (CD, EC 3.5 4.5) is a cytoplasmic enzyme primarily present in liver and polymorphonuclear leukocytes which catalyses the deamination of cytidine and its analogues to the corresponding uridine compounds. Serum level of cytidine deaminase have been shown to be raised in pre-eclampsia [1] and rheumatoid arthritis [2]. Measurement of cytidine deaminase activity has been used in the monitoring of patients with rheumatoid arthritis, when levels correlate with inflammatory activity.

0378-4347/89/$03 50 © 1989 Elsevier Science Publishers B V

Several colorimetric methods exist for the determination of cytidine deaminase activity Jones and Roberts [3] have described a method that utilises the Berthelot reaction to determine the ammonia produced by the deamination of cytidine, during an incubation of 18 h at 22°C Thompson et al. [4] have adapted this method to allow a more rapid analysis with an incubation of 4 h at 37°C Another kinetic method [5] measures the oxidation of NADH during the enzymic amination of α-ketoglutarate

Recently, high-performance liquid chromatographic (HPLC) methods have been developed that determine the formation of uridine as a measure of enzyme activity Three published methods have involved the use of isocratic reversed-phase HPLC to separate substrate and product of the enzymic reaction [6–8] These in general have optimised the conditions for separating cytidine and uridine, producing a rapid, reproducible assay with a high degree of sensitivity

Further experience with our published method [8] has shown it to be satisfactory for determining the relatively high levels of uridine formed in rheumatoid synovial fluid, after incubations of only 10 min However, problems arise when measuring the low levels seen in serum samples The reason is that using simple reversed-phase chromatography on octadecylsilane (ODS) columns affords poor resolution between uridine and the more frontally eluting cytidine, and therefore, when working with low enzyme activities and high detector sensitivity, the uridine peak is usually eluted on the tailing edge of a large cytidine peak Extending the incubation time at 56°C to increase production of uridine to overcome this problem not only increased the overall analysis time but also caused some loss of enzyme activity and denaturated some serum proteins The same criticism should apply to all the other published HPLC methods Another problem encountered when working at high detector sensitivity was the presence of UV-absorbing contaminants in the trichloroacetic acid (TCA) used for protein precipitation These prevented absorption reading at 262 nm, the maximum for uridine, thus further reducing the sensitivity

Analytical conditions for such trace chromatography have been given by Kirkland [9] For improved resolution, increased sensitivity and accurate quantitation it is preferable that the minor peak elutes prior to the major peak To satisfy these criteria it would be necessary to reverse the order of elution of the two compounds of interest. This paper describes the use of ion pairing to modify column selectivity and other minor changes to increase the sensitivity of the cytidine deaminase assay

EXPERIMENTAL

HPLC equipment

The chromatographic system consisted of an ACS LC500 pump (Applied Chromatography Systems, Macclesfield, U K.) and a Cecil CE 2012 variable-

wavelength UV detector (Cecil Instruments, Cambridge, U K) A stainless-steel column (100 mm × 4.6 mm I.D) was packed in house with 5-μm Hypersil ODS (Shandon Southern Products, Runcorn, U K). Samples were loaded by means of a Rheodyne 7125 injection valve with a 20-μl loop (Anachem, Luton, U K) Output from the spectrophotometer was recorded using a Spectra-Physics 4270 integrator (Spectra-Physics, St Albans, U K)

Chemicals

Cytidine, uridine and allopurinol were obtained from Sigma (Poole, U K), perchloric acid (60%, v/v), ammonium acetate, potassium dihydrogenphosphate (KH_2PO_4), hydrochloric acid (HCl), all analytical reagent grade, were obtained from BDH Chemicals (Poole, U.K), 1-octanesulphonic acid (OSA) was obtained from Eastman Kodak (Liverpool, U K). Water was glass-distilled and freshly deionised prior to use

Mobile phase

The mobile phase consisted of 100 m*M* ammonium acetate containing 1% (v/v) methanol and 1 m*M* OSA, adjusted to pH 5 0 with 6 *M* HCl. The mobile phase flow-rate was 1 2 ml/min

Serum samples

Whole blood was collected from patients attending routine rheumatology clinics into plain glass vacutainers and clotted at room temperature Serum was prepared by centrifuging at 1500 *g* for 10 min, aliquoted into 2-ml vials (Sterilin, Hownslow, U K) and stored at −20°C until required

Preparation of samples

Serum samples (100 μl), cytidine (0 8 m*M* in 50 m*M* KH_2PO_4, pH 7 0) and 50 m*M* KH_2PO_4 (pH 7.0) were preincubated at 56°C for 5 min. Cytidine (400 μl) was added to the serum sample (100 μl), which was mixed and incubated in capped tubes for 20 min at 56°C. Aliquots (400 μl) of the incubant were then removed and added to 100 μl of cold 6% (v/v) perchloric acid containing 0 75 m*M* allopurinol (internal standard) The sample was immediately mixed on a vortex mixer, then 500 μl of ammonium acetate (100 m*M*, pH 7 0) were added The sample was then centrifuged at 13 000 *g* in a Microcentaur (MSE, Crawley, U.K.) centrifuge for 10 min. The supernatant was removed and a 20-μl aliquot was injected onto the HPLC column Uridine production was calculated by reference to a uridine standard (0 1 m*M*), prepared in the same manner with correction for the internal standard concentration

RESULTS

Effect of ion-pair concentration on the retention of cytidine

Experiments were carried out to determine the effect of OSA on the reversed-phase chromatography of uridine, cytidine and allopurinol The concentration of ion pair was varied from 0 5 to 5 mM at a constant pH of 5 0 There was no demonstrable effect of ion pair on the k' values of allopurinol or uridine, however, cytidine was extremely responsive to increases in OSA concentration, being maximally retained at an OSA concentration of 2 5 mM. Further increases in ion-pair concentration resulted in a decrease in k' values for cytidine (Fig 1)

Effect of pH on the ion pairing of cytidine

Experiments were carried out with a circulating buffer of 100 mM ammo-

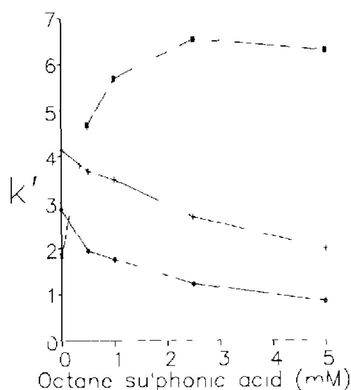

Fig 1 Effect of OSA concentration in HPLC eluent on the retention of cytidine (■), uridine (◇) and allopurinol (+)

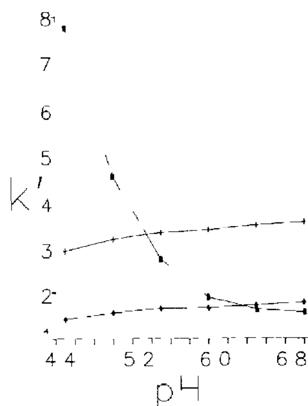

Fig 2 Effect of pH of eluent on the retention of cytidine (■), uridine (◇) and allopurinol (+)

nium acetate containing 1% (v/v) methanol and 1 mM OSA, and the pH gradually varied between 7 0 and 4.5 by the addition of 6 M HCl A concentration of OSA of 1 mM was used as this retained cytidine sufficiently and allowed a rapid analysis The ion pairing of cytidine with OSA was pH-dependent It was found that the retention of cytidine was extremely responsive to ion pairing at pH < 6 (Fig 2) With a pH of 6 cytidine eluted frontally, and when the pH was decreased towards 4 then cytidine was increasingly retained on the column A pH < 4 considerably increased the retention of cytidine The alterations in pH had no marked effect on the k' values of uridine or allopurinol

Chromatographic conditions

From the above investigations into the effect of ion-pair concentration and pH variations on the retention of uridine, allopurinol and cytidine, a chromatographic system was devised that would enable the criteria of Kirkland [9] to be satisfied The conditions chosen were 100 mM ammonium acetate with 1% (v/v) methanol and 1 mM OSA adjusted to a pH of 5 0 This provided a rapid separation of uridine, allopurinol and cytidine with sufficient retention to avoid frontally eluting UV-absorbing serum components (Fig 3) At max-

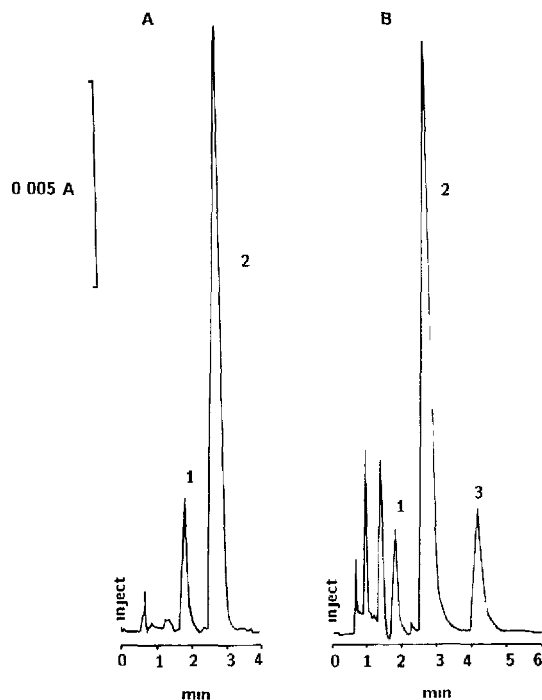

Fig 3 (A) Chromatogram of standards (B) Chromatogram of a serum sample after 20 min incubation at 56 °C Eluted from column with 100 mM ammonium acetate containing 1% (v/v) methanol with 1 mM octanesulphonic acid, pH 5, at a flow-rate of 1 2 ml/min and detected at 262 nm Peaks 1 = uridine, 2 = allopurinol, 3 = cytidine

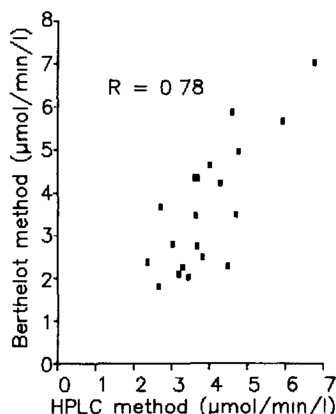

Fig 4 Correlation between cytidine deaminase activity in rheumatoid arthritic sera assayed using modified Berthelot and HPLC methods Mean (±S D) levels for the samples were 3 62±1 43 µmol/min/l for the Berthelot method and 3 94±1 07 µmol/min/l for the HPLC method (r=0 78, p<0 001) Line of best fit, y=1 06x−0 55

imum sensitivity picomolar levels of uridine could be measured The integrator response (peak area) was linear to 0.3 mM uridine (r=0 99) The rapid analysis time allowed a throughput of twelve samples per hour.

Reproducibility of the assay

Repeated injection of a uridine standard gave a coefficient of variation of 2.5% (n=10) A single serum sample was repeatedly assayed for cytidine deaminase activity as described in the methods: the mean (±S.D.) values were 3 70±0 29 µmol/min/l. The intra-assay coefficient of variation was 7 8% (n=10)

Correlation with existing colorimetric methods

Sera from twenty rheumatoid arthritic patients were assayed using both the HPLC assay and a modified Berthelot assay [4]. The mean (±S D) levels for the samples were 3 94±1.07 µmol/min/l for the HPLC method and 3.62±1 43 µmol/min/l for the colorimetric method The correlation between the two methods was r=0 78 (p<0.001) (Fig. 4)

DISCUSSION

The lack of resolution associated with previously published isocratic reversed-phase HPLC methods arises from the fact that cytidine is a polar compound and as such is poorly retained on a reversed-phase ODS column Uridine is only slightly retained, and these two facts lead to a poorly resolved chromatogram when isocratic reversed-phase conditions are employed. Therefore when working analytically with this system, low levels of uridine are difficult

to detect, when the sample contains high levels of cytidine In any enzyme assay the substrate is present in such concentrations to produce a V_{max} response and to promote a unidirectional reaction Therefore the problem outlined above will occur when using simple reversed-phase HPLC to determine cytidine deaminase activity

Most nucleosides and bases are readily separated by HPLC on the basis of their differing hydrophobicities [10], few workers have needed to introduce additional variables into their chromatographic systems, such as ion pairs or tertiary solvents However, Erhlich and Erhlich [11] found difficulty in separating eukaryotic DNA bases using simple reversed-phase chromatography Four of the DNA bases (cytosine, thymine, guanine and 5-methylcytosine) were not retained well on the column, due to their high polarity and as such the resolution was impaired. In order to improve the resolution they introduced the ion-pairing reagent heptanesulphate to their chromatographic system

Since cytidine (pK_a 4.22) is moderately ionised at the pH used, and both uridine (pK_a 9.77) and allopurinol (pK_a 9.4) are not, we were able to produce an ion-pair reversed-phase HPLC separation of these three compounds In our system the concentration of ion pair and the pH of eluent are such that cytidine is retained sufficiently, allowing uridine to elute frontally followed by allopurinol

Another improvement to the previously published method for cytidine deaminase is the use of perchloric acid (PCA) instead of TCA as a protein-precipitating agent, which enables uridine to be detected at its wavelength of maximum absorption (262 nm). When using TCA as a protein precipitant, uridine had to be detected at 280 nm due to the presence of a strongly UV-absorbing component in the TCA

The results obtained using this HPLC assay are comparable with those obtained using a standard spectrophotometric method However the HPLC assay has several advantages· (i) it has a greater sensitivity (picomole levels of uridine can be measured), (ii) it is more specific, assaying for uridine, a direct product of cytidine deaminase, whereas ammonia (Berthelot method) may come from other sources leading to a high background, (iii) it allows rapid analysis of serum samples (twelve per hour)

In conclusion the use of OSA as an ion-pairing agent enables detector sensitivity to be increased as uridine elutes alone frontally The use of PCA as a protein precipitant further increases the sensitivity, producing a rapid, more sensitive and reproducible HPLC assay for serum cytidine deaminase

REFERENCES

1 D D Jones, S Bahrjn, E L Roberts and G F Williams, Br J Obstet Gynaecol, 89 (1982) 314

2 P W Thompson, D D Jones and H L F Currey, Ann Rheum Dis, 45 (1986) 9

3 D D Jones and E L Roberts, Enzymes of DNA Metabolısm ın Clınıcal Dıagnosıs, Chancery Publıcatıons, Aberystwyth, 1984
4 P W Thompson, I T James, S Wheatcroft, R Pownall and C G Barnes, Ann Rheum Dıs, 48 (1989) 502
5 L Targett-Adams, D D Jones and G F Wıllıams, Clın Chım Acta, 63 (1975) 377
6 T Russo, Sel Top Clın Enzymol, 2 (1984) 17
7 D A Rıchards, R A Sherwood, D Ndebele and B F Rocks, Bıomed Chromatogr, 2 (1988) 48
8 K Herbert, D Perrett and D L Scott, J Pharm Bıomed Anal, 7 (1989) 737
9 J J Kırkland, Analyst, 99 (1974) 859
10 D Perrett, ın C K Lım (Edıtor), HPLC of Small Molecules, IRL Press, Oxford, 1986, p 221
11 M Ehrlıch and K Ehrlıch, J Chromatogr Scı, 17 (1979) 531