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Determination of cytidine deaminase activity in synovial fluid by HPLC

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Abstract: A reversed-phase high-performance liquid chromatographic method for the determination of cytidine deaminase activity in synovial fluid is described. Diluted synovial fluid was incubated for 10 min at 56°C with 0.4 mM cytidine. The protein in 5 vol of incubate was then precipitated using 1 vol of trichloroacetic acid (20% m/v) and the substrate, cytidine, and the product, uridine, were determined in the resultant supernatant. These substances were separated by reversed-phase HPLC using 0.05 M potassium dihydrogen orthophosphate (pH 6.5) containing methanol (3% v/v) and were detected at 280 nm. The enzyme activity was determined by measuring uridine formation. The effects of substrate concentration, pH and reaction temperature on uridine formation are described.

Keywords: *Cytidine deaminase; nucleoside deaminase; synovial fluid; reversed-phase high-performance liquid chromatography.*

Introduction

Cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5) is both a salvage and a degradative enzyme of pyrimidine nucleoside metabolism [1]. It catalyses the hydrolytic deamination of cytidine, deoxycytidine and a variety of their pharmacological analogues. *In vivo*, uridine and deoxyuridine are synthesized from cytidine and deoxycytidine, respectively (Fig. 1). The enzyme is widely distributed in human tissues, the highest levels being in liver, placenta, lung and neutrophils [2].

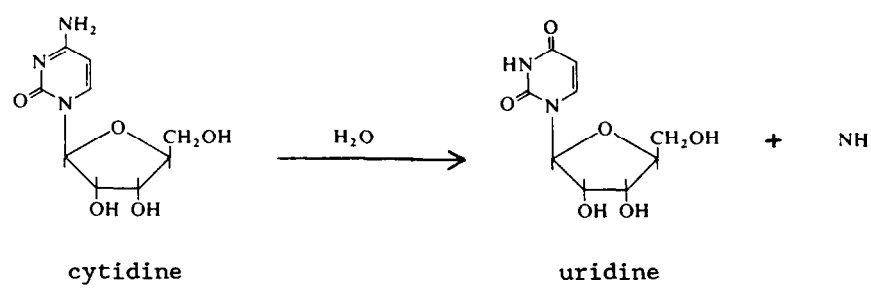

Figure 1
The enzymatic deamination of cytidine.

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Clinical interest in the enzyme began with the observation of its relevance to the development of resistance to the anti-cancer drug, cytosine arabinoside, in humans [3]. More recently, however, cytidine deaminase, since it is a cytosolic enzyme, has been used as a marker of cell damage in two diseases. It has been suggested that raised levels of serum cytidine deaminase are indicative of pre-eclampsia in pregnancy [4] although recently this has been disputed [5]. In the serum and synovial fluid of patients with rheumatoid arthritis the enzyme is a marker of acute inflammation [6]. The authors' interest in cytidine deaminase arises from its potential as a biochemical measure in the assessment of the progression of rheumatoid disease.

Previous assays for cytidine deaminase measured the decrease in absorbance at 290 nm [3] or the formation of radiolabelled uridine from [2-^{14}C]cytidine followed by paper chromatography and scintillation counting [7, 8]. Such assays suffer from lack of specificity or require the use of costly radiochemicals. An assay based on the detection of the ammonia generated by the deamination of cytidine has been used for large groups of clinical specimens [4]. However, the assay is time-consuming and relatively non-specific; in addition the product assayed, ammonia, is labile under the incubation conditions. Nucleosides such as cytidine and uridine are readily separated by HPLC using reversed-phase columns with simple buffer–methanol elution [9]. Furthermore, cytidine deaminase is reported to be very stable to heat [8]. Therefore, a rapid and specific assay has been developed by utilizing the heat stability of the enzyme and the specificity of HPLC with UV detection.

Experimental

Materials

The following materials were used: cytidine, uridine, uracil, phenol reagent, alkaline hypochlorite reagent, bovine serum albumin (Sigma, Poole, UK), potassium dihydrogen orthophosphate (KH_2PO_4), trichloroacetic acid (TCA), ammonium sulphate, sodium carbonate, tris(hydroxymethyl)aminomethane (Tris) and sodium bicarbonate (BDH, Poole, UK), HPLC grade methanol (Rathburn, Walkerburn, UK).

For the protein assay, stock solution **A** (Pierce UK Ltd, Cambridge, UK) consisted of 1% (m/v) disodium bicinchoninate, 2% (m/v) sodium carbonate monohydrate, 0.16% (m/v) sodium tartrate, 0.4% (m/v) sodium hydroxide and 0.95% (m/v) sodium bicarbonate in deionized water; the final pH was 11.25. Stock solution **B** consisted of 4% (m/v) copper sulphate in de-ionized water. Bicinchoninic acid working reagent was prepared daily by mixing solution **A** with solution **B** (50:1, v/v). A standard curve of bovine serum albumin, 0–60 $\mu g\ ml^{-1}$ in 10 $\mu g\ ml^{-1}$ steps was used. Absorbances at 562 nm were measured using a SP500 spectrophotometer (Pye-Unicam Ltd, Cambridge, UK).

Reversed-phase HPLC

Stainless-steel columns (100 × 4.6 mm i.d.) were packed in-house with 3-μm ODS Hypersil according to the manufacturer's instructions (Shandon Southern, Runcorn, UK). HPLC separations were performed with a 2150 double reciprocating piston pump (LKB, Croydon, UK) and a syringe-loaded valve (Rheodyne 7125, 20-μl loop); peaks were detected at 262 or 280 nm with a CE2012 variable wavelength UV monitor (Cecil Instruments, Cambridge, UK; 8-μl flow cell). Peak areas were determined using a SP4270 integrator (Spectra Physics, St Albans, UK).

All separations on the 3-μm ODS Hypersil column were performed at ambient temperature by isocratic elution (1.2 ml min^{-1}) with 0.05 M KH_2PO_4 (pH 6.5) containing methanol (3% v/v).

Sample preparation

Samples of synovial fluid, where therapeutically indicated, were collected from patients with rheumatoid arthritis or osteoarthritis and centrifuged at 1000 ***g*** for 15 min to remove cells and debris. Aliquots of supernatant were stored at −20°C until analysis.

Assay procedure

Diluted plasma or synovial fluid (1 ml; 20% v/v in 0.05 M KH_2PO_4, pH 7.0) was pre-heated to 56°C for 10 min. The reaction was initiated by adding 1 ml of pre-warmed cytidine solution (0.8 mM in 0.05 M KH_2PO_4, pH 7.0). The reaction was stopped (0 min and 10 or 60 min) and protein precipitated from 0.5-ml aliquots by the addition of 0.1 ml of trichloroacetic acid (20% m/v). Following centrifugation at 3000 ***g*** for 10 min, an aliquot of supernatant (0.4 ml) was mixed with an equal volume of 0.35 M KH_2PO_4 (pH 7.0) to adjust the pH to approximately 6.5. Samples (20 μl) were injected into the chromatograph. In order to minimize a minor source of interference present in the TCA solution, which was eluted as a broad peak at wavelengths below 275 nm, the detector was set at 280 nm.

Identification and determination. Under these HPLC conditions, uracil, cytidine and uridine standards were fully resolved; retention times were 1.6, 2.0 and 2.6 min, respectively. Peaks in unknowns were identified by comparison of retention times and absorbance ratios (280/262 nm) versus these standards and by "spiking" with small amounts of standard (50 μM) and detecting increases in peak area. The dilution of synovial fluid required in the assay (1:24 v/v) and the wavelength (280 nm) meant that no interfering peaks were present in blanks (no cytidine added) at the sensitivity used (see Results). Interference with cytidine quantification by co-elution with hypoxanthine and xanthine is therefore not seen because of the low absorbances of these compounds at 280 nm. The relative standard deviation (RSD) for the determination of cytidine added to synovial fluid was 4.8%.

Calculation of cytidine deaminase activity. Cytidine deaminase activity was calculated by measurement of uridine formation. The amount of uridine formed per 10 min incubation was calculated by comparison of peak area with that of standard uridine (0.2 mM). After allowing for dilution factors the activity was expressed as μmol uridine formed per litre of synovial fluid min^{-1}.

Cytidine deaminase assay by the determination of ammonia

For comparison with the HPLC method, samples of synovial fluid were assayed for cytidine deaminase activity using the Chaney and Marbach [10] modification of the Berthelot ammonia determination. The procedure was that of Jones *et al.* [4] but using a series of ammonium sulphate standards of 0–0.5 mM (in 0.1 M sodium carbonate–bicarbonate buffer) in 0.1 mM steps. Synovial fluid samples were analysed initially at a 1:10 dilution (v/v in carbonate/bicarbonate buffer) and, if required, analyses were repeated using other dilutions.

Protein assay

The protein content of supernatants formed by TCA precipitation of diluted synovial fluid (1:10 v/v with 0.05 M KH_2PO_4, pH 7.0) was determined by the bicinchoninic acid (BCA) procedure of Smith *et al.* [11] but with modifications to increase sensitivity. The final procedure was as follows. Bovine serum albumin standards (0–60 μg ml^{-1}) and TCA supernatants (diluted 1:25 v/v with de-ionized water) were mixed in duplicate (200 μl) with 1 ml of BCA working reagent. The mixture was then heated at 60°C for 30 min and the absorbance measured at 562 nm using a SP500 spectrophotometer.

Results and Discussion

Extraction of nucleosides from synovial fluid

To the authors' knowledge, the efficiency of protein removal from synovial fluid by common deproteinizing techniques has never been addressed. Although the protein concentration of synovial fluid is only half that of plasma, it contains large amounts of hyaluronic acid which greatly increases the viscosity and may interfere with the precipitation of proteins. This is important for samples being analysed by HPLC since injection of large amounts of protein on to the column results in loss of column efficiency and in increases in pressure. Blanchard [12] has shown that TCA efficiently precipitates plasma proteins. Therefore, different amounts of TCA (20% m/v) were used to precipitate proteins from 0.5 ml of diluted rheumatoid arthritis synovial fluid (1:10 v/v with 0.05 M KH_2PO_4, pH 7.0). The supernatant was collected by centrifugation at 3000 ***g*** for 10 min. The efficiency of protein removal was evaluated by determining the protein content of supernatants (Table 1). Arbitrarily, an efficiency of >99% was chosen to be acceptable; thus 100 μl of TCA (20% m/v) was found necessary to achieve this level of protein removal.

These findings on the efficiency of TCA in removing proteins from synovial fluid are comparable with those reported by Blanchard [12] for plasma. Therefore, it is deduced that the presence of hyaluronic acid has little effect on protein precipitation by TCA.

Recovery of exogenous cytidine and uridine from synovial fluid using this protocol, compared with recovery from buffer alone, was 105 ± 2% (n = 6). This difference is probably due to the volume occupied by synovial fluid protein.

Table 1
The efficacy of protein removal from synovial fluid by TCA. Synovial fluid was diluted 1:10 (v/v) with 0.05 M KH_2PO_4 (pH 7.0) prior to addition of TCA

Volume TCA (20% m/v) added (μl) to 0.5 ml of diluted synovial fluid	Protein removed from synovial fluid (% m/m of total)
10	97.4
25	96.3
50	96.8
75	98.5
100	99.1
150	99.3

The results are expressed as the percentage synovial fluid protein precipitated and represent the mean of duplicates.

HPLC separation and peak quantitation

Optimal separation of a standard mixture of cytidine, uridine and uracil was achieved on a 3-μm ODS Hypersil column (100 × 4.6 mm, i.d.) eluted with 0.05 M KH_2PO_4 (pH 6.5) containing 3% (v/v) of methanol, at a flow rate of 1.2 ml min^{-1}. Peaks were detected by absorbance at 280 nm, and quantified using peak areas determined by integration (Fig. 2a). Although maximum sensitivity with respect to uridine detection was achieved at 262 nm, interference was noted when analysing TCA-treated samples at this wavelength. This was minimized by using a higher wavelength (280 nm; Fig. 2b); the sensitivity to uridine was still adequate for the purpose of the method. This also illustrates that endogenous synovial fluid components do not interfere with the quantitation of cytidine and uridine. Cytidine and uridine were identified by retention time and increase in peak area following addition of individual standards and peak height ratio ($A280/A262$ nm = 1.0 for cytidine and 0.5 for uridine). With this procedure the integrator response was linear up to 0.3 mM of either cytidine or uridine. The RSD for repeated injections of standards (0.125 mM) was 2%.

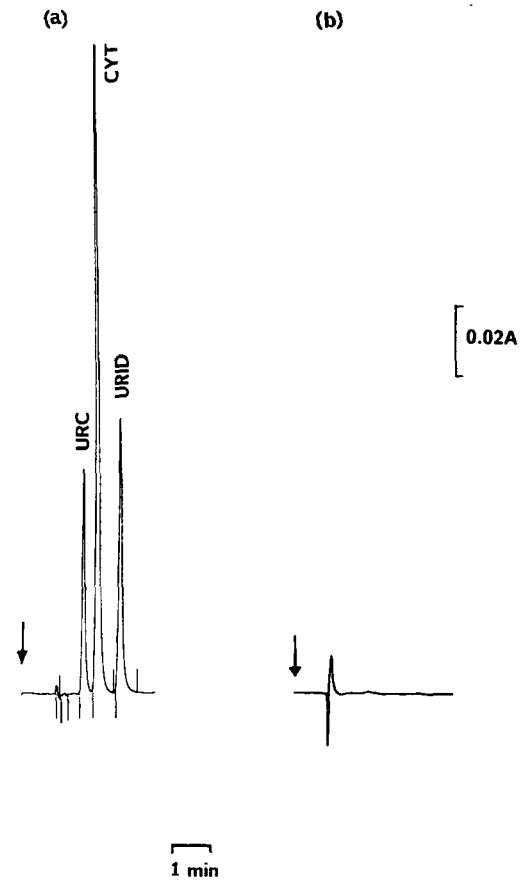

Figure 2
Reversed-phase HPLC separation of uracil, cytidine and uridine. (a) Standard compounds (0.2 mM), (b) TCA-extracted synovial fluid blank.

Enzyme assay

Cytidine deaminase is relatively heat stable [8] and elevated temperatures can be used to produce a more rapid assay. Following preliminary studies, a reaction temperature of 56°C was chosen; this was the highest pre-set temperature on a model FE water-bath (Grant Instruments, Cambridge, UK).

In a pilot experiment a specimen of synovial fluid from a patient with rheumatoid arthritis was diluted (1:12 v/v) with 0.05 M KH_2PO_4 (pH 7.0) containing cytidine (0.5 mM, final concentration). After incubation at 56°C for 1 h, approximately 50% of the cytidine had been converted into uridine as determined by HPLC (data not shown). Based upon this observation the authors aimed to develop the assay in diluted synovial fluid (1:10 v/v with buffer/substrate) at 56°C, measuring the amount of uridine formed in the first 10 min.

Under these conditions and at pH 7.2 the K_m and V_{max} for synovial fluid cytidine deaminase, based on the production of uridine, were 20 μmol l^{-1} and 3.2 μmol l^{-1} min^{-1}, respectively, using the Woolf Plot [13] over the range of cytidine concentration of 0.01–0.35 M. The K_m determined here (20 μmol l^{-1}) is greater than, but of the same order as, the values previously reported for human granulocytes (11 μmol l^{-1} [14]), human peripheral mononuclear cells (3.6 μmol l^{-1} [8]) and human liver (9.2 μmol l^{-1}; [15]. From the present data, a cytidine concentration of 0.4 M was chosen as this represented both substrate saturation and economy.

The effect of pH on cytidine deaminase activity in a pool of synovial fluid samples was determined over the range of pH 4.2–9.7. The incubation buffers were acetate (0.1 M), KH_2PO_4 (0.2 M), Tris–hydrochloride (0.2 M) and bicarbonate (0.1 M). The rate of uridine formation was plotted as a function of pH at 56°C (Fig. 3). A broad pH optimum was observed in line with previous studies using human leucocytes [16], human granulocytes [14], human mononuclear cells [8] and human liver [15]. The median value obtained, pH 7.0, was chosen for the assay.

Three consecutive incubations were used to study the effect of heat on the enzyme activity in pooled synovial fluid samples. Synovial fluid and substrate–buffer were heated separately at 37 or 56 or 70 or 100°C for 5 min prior to determination of cytidine deaminase activity. Each reaction was initiated by mixing equal volumes of synovial fluid and substrate solution and incubation continued at 37°C for all samples for a further 20 min. Uridine formation for each of the pre-reaction temperatures is shown in Table 2. Boiling (100°C) completely eliminated enzyme activity. Incubation at 70°C decreased the formation of uridine very slightly, but at 56°C the conversion was similar to that using 37°C throughout. These findings on the heat stability of cytidine deaminase agree with those of previous studies on human leucocytes [16], granulocytes [14], blood mononuclear cells [8] and liver [17].

The results of the assay for cytidine deaminase were linear ($r = 0.99$) up to 8 μmol uridine formed per litre of synovial fluid min^{-1} (data not shown). The RSD was 4.8%.

An example of the HPLC assay for cytidine deaminase in synovial fluid is shown in Fig. 4. By comparison with the chromatogram of standards (Fig. 2a), it can be seen that no uracil is formed from uridine by the side reaction of uridine–orthophosphate ribosyltransferase (EC 2.4.2.3) under these conditions. Furthermore, since there is unlikely to be ATP present in the incubation mixture, the synthesis of cytidine monophosphate and uridine monophosphate from cytidine and uridine, respectively, by uridine–cytidine kinase (ATP)–uridine 5′-phosphotransferase, EC 2.7.1.48) is not possible. If these considerations are taken into account together with the ability to

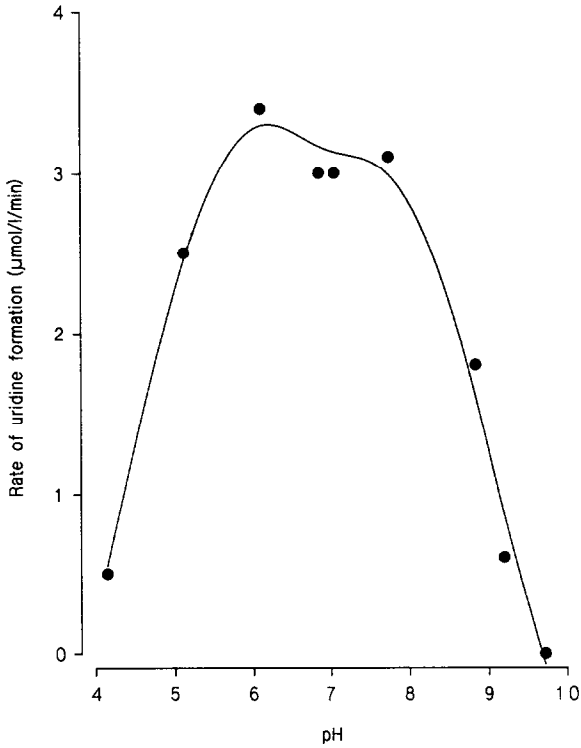

Figure 3
The effect of pH on the cytidine deaminase activity in synovial fluid.

Table 2
The heat stability of cytidine deaminase activity in synovial fluid

Pre-assay incubation temperature (°C)	Uridine formed (μmol l^{-1} min^{-1})
37	1.03
56	0.97
70	0.65

measure cytidine and uridine directly and without interference, it is concluded that the HPLC assay is a true measurement of cytidine deaminase activity.

Comparison of assays for cytidine deaminase

The HPLC assay was compared with an assay based on the Berthelot determination of ammonia [4]. Ten samples of synovial fluid ware analysed by both methods. There was a good correlation between the two methods (r = 0.99; Table 3).

Conclusions

A rapid and specific assay has been described for cytidine deaminase in synovial fluid by utilizing the heat stability of the enzyme and the specificity of HPLC with UV

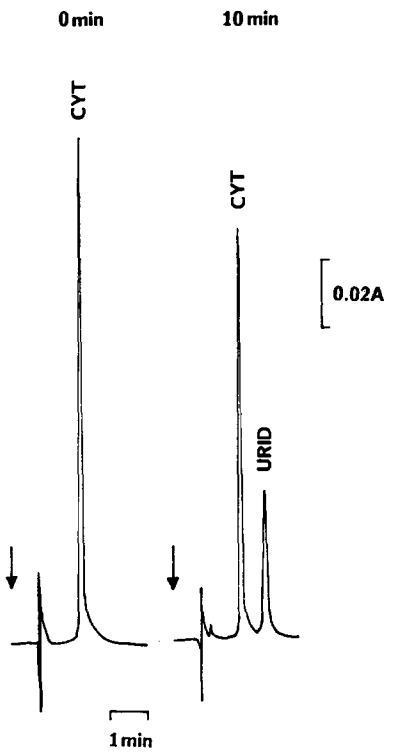

Figure 4
An example of the assay for cytidine deaminase in synovial fluid from patients with rheumatoid arthritis. URC, uracil; CYT, cytidine; URID, uridine.

Table 3
Comparison of the HPLC assay for cytidine deaminase with an assay based on the determination of ammonia generation

Synovial fluid no.	Berthelot (μmol $\times$ 10^{-4} of ammonia formed l^{-1} min^{-1} at 22°C)	HPLC (μmol of uridine formed l^{-1} min^{-1} at 56°C)
1	73.0	56.6
2	5.2	4.6
3	2.3	1.7
4	73.8	44.4
5	52.0	40.5
6	95.3	70.7
7	0.43	0.46
8	0.56	0.5
9	0.37	0.31
10	0.02	0.06

detection. This is, in effect, only a specific application of the well established chromatography of nucleosides and bases on reversed-phase packing materials.

With minor modifications the assay could also be used to determine the activity of cytidine deaminase in other biological fluids, for example plasma and serum.

With the HPLC procedure it is possible to analyse at least 15 samples per hour; therefore, even allowing for the incubation and sample processing time and using manual injection, some 60 samples can be processed per working day. The assay is being used as

a measure of synovial inflammation in the authors' studies on disease progression in rheumatoid arthritis.

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