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Short communication

Simultaneous separation by high-performance liquid chromatography of carbamoyl aspartate, carbamoyl phosphate and dihydroorotic acid

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Abstract

Leflunomide is an immunomodulatory drug which acts by inhibiting dihydroorotic acid dehydrogenase, the fourth enzyme of pyrimidine biosynthesis. We modified our high-performance liquid chromatography method to demonstrate that the principal metabolite in mitogen-stimulated human T-lymphocytes incubated with leflunomide was not dihydroorotic acid, but carbamoyl aspartate. Identification involved preparation of [^{14}C]carbamoyl aspartate from [^{14}C]aspartic acid and mammalian aspartate transcarbamoylase. Accumulation of carbamoyl aspartate indicates that under these conditions the equilibrium constant for dihydroorotase favours the reverse reaction. This HPLC method, enabling simultaneous separation of the first four intermediates in the de novo pyrimidine pathway may be of use in a variety of experimental situations. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Leflunomide; Brequinar; Dihydroorotic acid; Carbamoyl aspartate; Carbamoyl phosphate

1. Introduction

Leflunomide (LFM) is a novel drug with both anti-inflammatory and immunomodulatory properties. It has recently completed Phase III clinical trials in patients with rheumatoid arthritis, but its mode of action in vivo has been the subject of considerable debate [1–11]. Earlier studies using either the purified enzyme or lysed cell preparations showed that LFM resembles Brequinar (BQR) a known de novo pyrimidine synthesis inhibitor at the level of the 4th enzyme (Fig. 1), dihydroorotic acid dehydrogenase (DHODH). Both LFM and BQR were non-competitive inhibitors of DHODH and occupied the same site on the enzyme [6–11]. Other studies questioned the involvement of pyrimidine metabolism as the primary mode of action of LFM, inhibition of various tyrosine kinases being implicated

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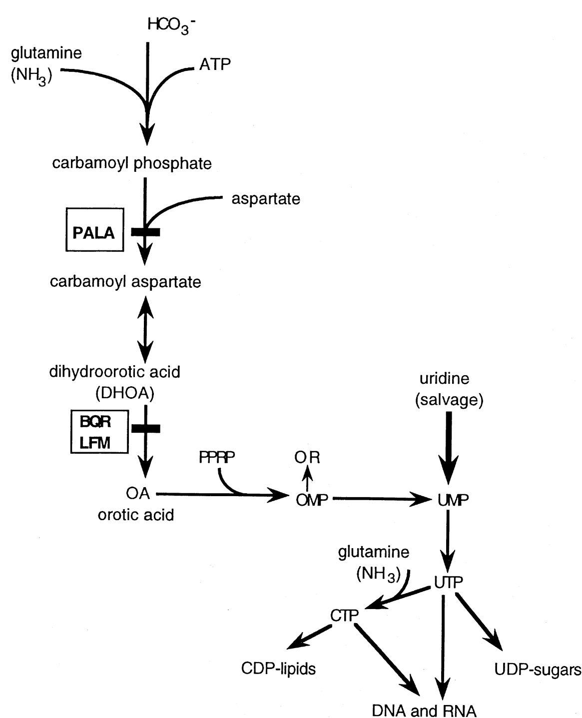

Fig. 1. Pathways of pyrimidine biosynthesis and salvage in human T-lymphocytes. The point at which [^{14}C]bicarbonate is incorporated is highlighted. The enzymes inhibited by Leflunomide (LFM), Brequinar (BQR) and *N*-phosphonacetyl-L-aspartate (PALA) are indicated. Abbreviations: DHOA, dihydroorotic acid; OA, orotic acid; OR, orotidine; OMP, orotidylic acid; PPRP, 5-phosphoribosyl-1-pyrophosphate.

from animal models [2,5]. Recently we published data which demonstrated conclusively that the prime target of LFM in vitro in human T-lymphocytes, as for BQR, was de novo pyrimidine synthesis [12]. Uridine rescue studies completely reversed the inhibitory effect of either drug on PHA induced T-lymphocyte proliferation and restored expansion of all ribonucleotide pools. However, uridine restored incorporation of [^{14}C]bicarbonate into purine pools only. Incorporation into pyrimidine pools was still restricted totally, radiolabel accumulating in an intermediate with the retention time (t_R) of dihydroorotic acid (DHOA), thereby confirming a block in de novo pyrimidine ribonucleotide biosynthesis at the level of DHODH [12].

The objective of the present study was to establish beyond doubt that the principal [^{14}C]metabolite accumulating in our human T-lymphocyte model in the presence of either LFM or BQR was indeed DHOA [12]. To achieve this we used three different HPLC methods developed by us, one of which was modified specifically for the purpose.

2. Experimental

2.1. Reagents and chemicals

PHA, trichloroacetic acid (TCA) and all chemicals and standards (Analar or Aristar grade), including DHOA, orotidine (OR) and orotic acid (OA), RPMI without bicarbonate and PHA, were purchased from Sigma (Poole, UK). [^{14}C]Bicarbonate (54 mCi/mmol) from Amersham Life Science (Little Chalfont, UK). RPMI-1640, Hanks balanced salt solution (HBSS), heat-inactivated foetal calf serum (FCS), penicillin/streptomycin (10 000 units/ml and 10 000 μg/ml, respectively) and 24-well plates were all obtained from Gibco Life Technologies (Paisley, UK).

Brequinar and A77 1726 (the active metabolite of Leflunomide) were synthesised by Hoechst Marion Roussel (Wiesbaden, Germany). *N*-Phosphonacetyl-L-aspartate (PALA) was obtained from the National Cancer Institute (Bethesda, MD, USA).

2.2. Synthesis of radiolabelled carbamoyl aspartate

Since the second intermediate in the de novo pyrimidine pathway, carbamoyl aspartate, is non-UV absorbing and no radiolabelled product was available commercially, this intermediate was synthesised as follows by a two-step process:

(a) *Preparation of mammalian aspartate transcarbamoylase*. The multienzyme polypeptide CAD [containing the activities carbamoyl phosphate synthetase (CPS), aspartate transcarbamoyl synthetase (ATCase) and dihydroorotase (DHO)] was prepared from an overproducing hamster cell line [13]. A fragment containing the mammalian ATCase was generated by digestion with trypsin followed by ion-exchange chromatography on DE-Sepharose

[14]. The purity and molecular size of the product (40 kDa as expected for mammalian ATCase) was confirmed by SDS–PAGE [15], and the activity established using a colorimetric enzyme assay [14].

(b) *The enzymatic synthesis of* [^{14}C]*carbamoyl aspartate* was performed in 0.1 *M* Tris buffer (pH 7.4) which included 0.1 *M* KCl, 1 m*M* dithiothreitol, 7.5% (v/v) dimethyl sulphoxide, 2.5% (w/w) glycerol, 5 m*M* aspartic acid (containing 0.5 μCi [^{14}C]aspartic acid), 1 m*M* carbamoyl phosphate [14]. A 50 μl volume of ATCase was added to 200 μl of assay buffer at 37°C and the reaction stopped at 30, 45 and 60 min by adding 25 μl of 40% TCA, which was back-extracted with water-saturated diethylether to a pH of 5.0.

2.3. T-lymphocyte studies using de novo synthesis inhibitors

All experiments were carried out using pure T-lymphocytes from healthy humans, isolated as described [12] and incubated in full medium (RPMI-1640 supplemented with antibiotics plus 10% heat-inactivated, *dialysed* FCS). Briefly 1×10^{6} pure T-cells supplemented with 2–3% peripheral blood mononuclear cells were preincubated in 0.9 ml of full medium either alone, or containing LFM (25 μ*M*), or BQR (1 μ*M*) [12]. After 30 min T-cells were stimulated with 0.1 ml of 50 μg/ml PHA and incubated at 37°C for up to 72 h [12]. In separate experiments T-cells were preincubated with 1 m*M* PALA, which inhibits the second enzyme of pyrimidine biosynthesis, aspartate transcarbamoylase (ATCase, Fig. 1), prior to stimulation. PALA was added also after 24 h to cultures of stimulated T-cells preincubated with 1 μ*M* BQR and the incubation continued for a further 48 h.

2.4. Pulse-labelling radiotracer studies

At 24 h intervals T-cells were centrifuged, washed and incubated with [^{14}C]bicarbonate (1.1 m*M*) at 37°C for 2 h in fresh medium, with or without inhibitors, in sterile tightly capped 1.5 ml Eppendorf tubes. Reactions were stopped by centrifugation. Protein was precipitated from cell and medium using TCA and the latter back-extracted using water-saturated diethylether [12,16].

2.5. High-performance liquid chromatography (HPLC) systems

Extracts of cells and medium were processed using a Waters Trimodular system incorporating in-line photodiode array analysis at 254 and 280 nm coupled with radio-detection (Reeve Analytical, UK) [16–18]. Retention times (t_R) were verified using standards of known concentration [16,17]. Quantification of components eluting in the three separate HPLC systems employed was coupled with simultaneous monitoring of the rate and route of incorporation of radiolabel as follows:

System 1: Anion-exchange HPLC system for the separation of nucleotides. Briefly 100–175 μl of T-cell extract was injected onto a Phenomenex Hypersil 5 μm NH2-2 column (250×3.2 mm). Nucleotides were eluted using a linear gradient (buffer A, 5 m*M* KH_2PO_4; buffer B, 0.5 *M* KH_2PO_4–1.0 *M* KCl, initial pH 2.65 and 3.5, respectively) and a flow-rate of 0.5 ml/min [12,16].

System 2: Reversed-phase HPLC system for analysis of nucleosides and bases in cell extracts and medium. Extracts (10–100 μl) of the above cells and medium were injected onto a reversed-phase system consisting of a Phenomenex 5 μm ODS column (250×3.2 mm) [17]. UV absorbing components were eluted over 35 min at a flow-rate of 0.5 ml/min using a linear gradient (100% buffer A 50 m*M* ammonium acetate pH 5.0 to 30% buffer B, methanol–acetonitrile–tetrahydrofuran, 80:10:10).

System 3: Separation of pyrimidine de novo intermediates. Extracts of cell and medium were processed subsequently using a modification of an isocratic HPLC system developed for measuring orotic acid (OA) and orotidine (OR) in biological fluids [18]. The column employed was a Spherisorb S5 SAX 5 μm column (250×4.8 mm). UV absorbing components were eluted isocratically at a flow-rate of 1 ml/min (run time 15 min) with formic acid buffer containing 1.23 *M* formic acid adjusted to pH 2.3. The advantage of this method was that nucleotides, nucleosides and bases other than OR or QA eluted together in the first 4 min. Omission of the methanol from the buffer [18] enabled complete

separation of DHOA from OA and OR by nearly 6 min.

3. Results and discussion

Extracts of mitogen-stimulated human T-lymphocytes incubated with LFM or BQR and pulse-labelled with [^{14}C]bicarbonate were processed first on System 1, the anion-exchange HPLC system used previously [12]. The results confirmed that these two inhibitors of DHODH, the enzyme catalysing the fourth step of pyrimidine biosynthesis, completely blocked incorporation of radiolabel into the nucleotides UTP and CTP from 24 h onwards [12]. In the presence of LFM and BQR radiolabel accumulated instead principally in a peak with a t_R value of approximately 10 min (referred to here as 'DHOA') corresponding to that of a standard of pure DHOA (data not shown). Since DHOA, OA and OR all had similar t_R values in this anion-exchange system, as did IMP (9–10 min), we carried out further studies to confirm the identity of the 'DHOA' accumulating.

The same T-lymphocyte extracts were processed next using System 2, the reversed-phase HPLC system developed specifically for separating nucleosides and bases [17]. Although DHOA and OA were still not separated on this system, OA and OR had different t_R values, as did IMP. Again the principal radiolabelled metabolite eluted in a peak with the t_R value anticipated for a pure standard of DHOA (not shown), suggesting this metabolite was indeed DHOA.

Finally, we tested System 3, an HPLC method developed originally to separate OA and OR from other nucleosides and bases in human urine [18]. A slight modification of this method produced an excellent separation of DHOA from other nucleosides and bases as well as OA and OR (Fig. 2A and B). The t_R value for DHOA was approximately 5.1 min, 1 min later than all the other purine and pyrimidine nucleosides and bases in a standard mixture (Fig. 2B), which eluted together between 2 and 4 min, the t_R values for OA being 11 min, OR 12.2 min. All nucleotides (principally ATP, ADP, GTP and NAD in human erythrocytes) also eluted between 2 and 4.4 min; i.e. earlier than DHOA (Fig. 2C). UTP and CTP standards had the same t_R values as the purine nucleotides (not shown). The clear separation of DHOA from all these nucleotides was confirmed by spiking the same erythrocytes used in Fig. 2C with DHOA (Fig. 2D). The t_R value of DHOA in the spiked erythrocyte extract was approximately 5.1, the same as for the pure standard (Fig. 2A).

When the extracts of the T-cells stimulated alone with PHA and pulse-labelled with [^{14}C]bicarbonate were processed using this modified HPLC system coupled with in-line radiodetection, radiolabel eluted in two major peaks with t_R values of 3.4 and 3.9 (Fig. 3A). The radiolabelled peak at 3.9 min contained the coeluting purine and pyrimidine nucleotides. A minor peak also eluted at 4.6 min (N.B. the 0.8–0.9 min delay in t_R values compared with the UV traces in Fig. 2 is due to the time of transit through the radiodetector). In the T-cell extracts preincubated with either LFM (Fig. 3B), or BQR (Fig. 3C) the radiolabelled peak at 4.6 min increased, while the radiolabel in the peak containing the coeluting nucleotides at 3.9 min was reduced. The much smaller radiolabelled peak eluting at 5.9–6.0 min would correspond with the t_R value anticipated for [^{14}C]DHOA. Thus, the main radiolabelled peak at 4.6 min accumulating in the T-lymphocytes incubated with LFM or BQR was clearly not radiolabelled DHOA, or nucleotides.

To establish whether the peak at 4.6 min corresponded to an earlier non-UV absorbing intermediate of the de novo pyrimidine pathway, T-lymphocytes were preincubated with 1 m*M* PALA (which inhibits aspartate transcarbamoylase (ATCase, Fig. 1) and extracts processed using the same modified HPLC method. Radiolabel in these experiments accumulated in peaks with t_R values of 3.4 and 3.9 min, corresponding to bicarbonate and carbamoyl phosphate, respectively (Fig. 3D). Importantly, the principal radiolabelled peak in the T-cells incubated in the presence of BQR alone and extracted at 24 h eluted at 4.6 min, but in the extract of the same T-cells made at 48 h to which PALA had been added at 24 h, the t_R value of the major peak changed from 4.6 to 3.9 min (Fig. 3E). Only a small amount of radiolabel now eluted at 4.6 min, confirming that the first of the two radiolabelled peaks eluting in the T-cells incubated with BQR alone, with a t_R value of 3.9 min (Fig. 3C), was carbamoyl phosphate.

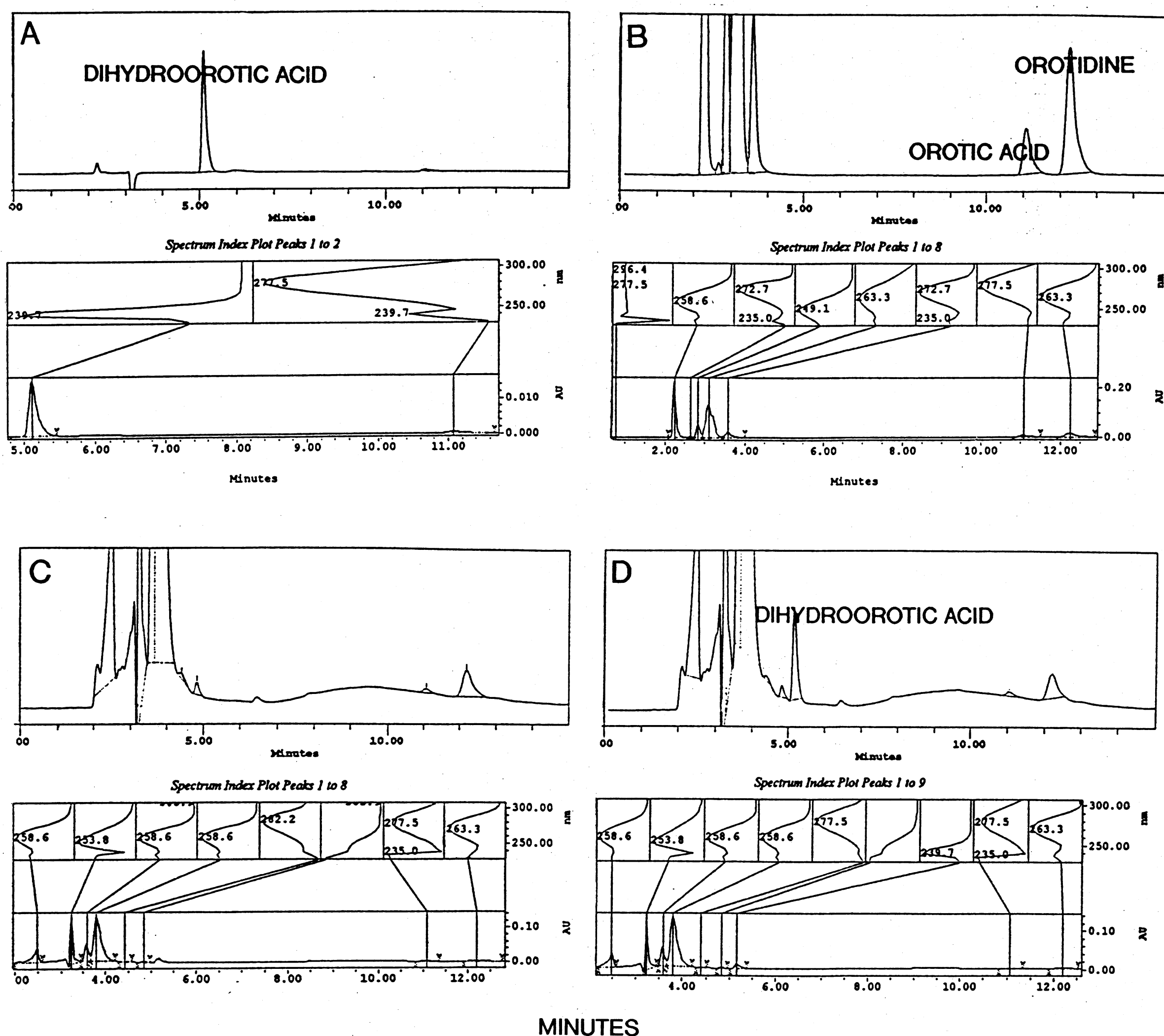

Fig. 2. Chromatograms obtained using System 3 at 254 nm and 0.5 AUFS (upper panel) with in-line diode array analysis from 230–310 nm (lower panel) confirming peak identity by the characteristic UV spectra recorded following injection of: (A) 10 μl of a DHOA standard showing a t_R value of 5.1 min; (B) 10 μl of a mixture of 16 pyrimidine and purine nucleosides/bases [17], showing that all are eluted by 4 min, except for orotic acid (OA), orotidine (OR) and DHOA; (C) 75 μl of an erythrocyte extract. Note the absence of any peak at 5.1 min and the presence of OR, together with a trace of OA. (This extract was from a patient treated with allopurinol where OR accumulates [17]); (D) 75 μl of the same erythrocytes spiked with DHOA prior to extraction. Note that all the nucleotides (principally ATP) were eluted by 4.4 min, giving a clear separation from DHOA, which eluted at 5.1 min.

The above findings suggested that the major radiolabelled peak accumulating at 4.6 min in the T-cells preincubated with either LFM or BQR (Fig. 3B and C), must be carbamoyl aspartate. The identity of the peak eluting at 4.6 min was established by injecting various concentrations of the [^{14}C]carbamoyl aspartate synthesised for the purpose onto the Spherisorb S5 SAX column. In each instance a single radiolabelled product eluted with a t_R value of 4.6 min (Fig. 3F), identical to the major peak in the T-cells preincubated with LFM or BQR (Fig. 3B and C). These results indicated that DHOA (the small peak at ~6.1 min with the t_R value of [^{14}C]DHOA, Fig. 3B and C) was clearly only a

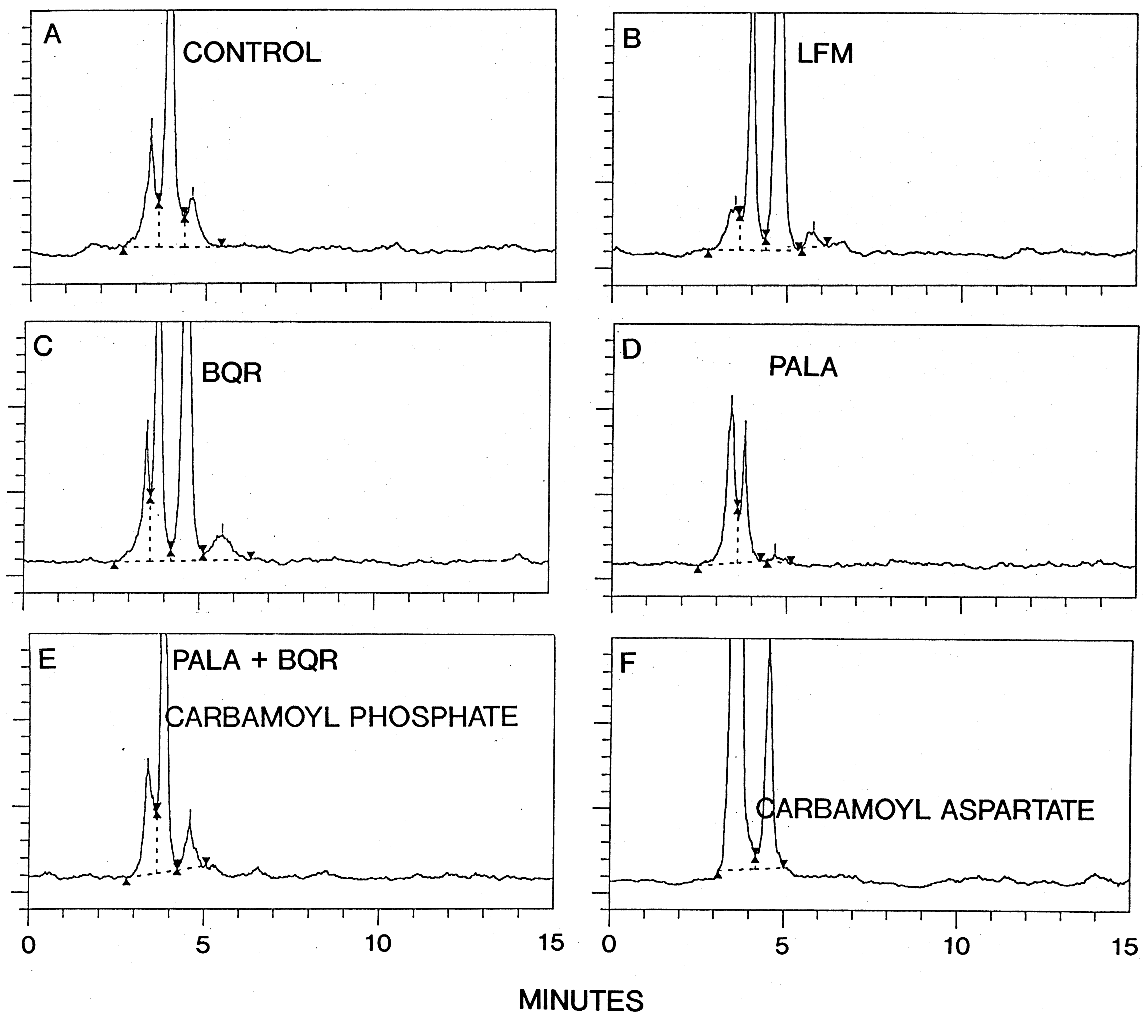

Fig. 3. Radio-traces of extracts of PHA stimulated T-cells pulse-labelled for 2 h with [^{14}C]bicarbonate made at the times shown after stimulation with PHA. (A) 200 μl of T-cells incubated for 72 h alone, or (B) preincubated with 25 μm LFM; (C) 200 μl of extract at 24 h of T-cells preincubated with 1 μm BQR; (D) 200 μl of extract made at 48 h of T-cells preincubated with 1 m*M* PALA; (E) 200 μl of extract T-cells preincubated with 1 μm BQR at zero time (as in C), to which 1 m*M* PALA was added at 24 h and incubated for a further 24 h, show that the peak accumulating at 3.9 min is carbamoyl phosphate; (F) 10 μl of the extract made following incubation of [^{14}C]aspartic acid with pure ATCase and stopped at 60 min, confirming that the largest peak accumulating in B and C with a t_R value of 4.6 min is carbamoyl aspartate.

minor metabolite in the T-cells incubated with these DHODH inhibitors. The possibility that DHOA accumulating might have been excreted into the incubation medium was excluded by processing the medium from all the above experiments on the same three HPLC systems (not shown). Further confirmation of the identity of 'DHOA' as carbamoyl aspartate was obtained by injecting the same synthetic [^{14}C]carbamoyl aspartate onto the anion-exchange HPLC System 1. [^{14}C]Carbamoyl aspartate eluted at approximately 10 min, i.e. with the same t_R value of the principal radiolabelled intermediate accumulating in the T-cells [12] preincubated with LFM or BQR (not shown).

In summary, these results demonstrate that although the target enzyme in mitogen-stimulated

human T-lymphocytes incubated with the immunomodulatory agents LFM and BQR is DHODH [12], DHOA is not the main metabolite accumulating. Development of a modified HPLC method, coupled with preparation of [^{14}C]carbamoyl aspartate from [^{14}C]aspartic acid and mammalian aspartate transcarbamoylase, confirmed the principal metabolite as carbamoyl aspartate. Accumulation of carbamoyl aspartate, not DHOA as anticipated, demonstrates that the reversible reaction catalysed by dihydroorotase [19,20] favours the accumulation of carbamoyl aspartate in healthy human T-cells under physiological conditions when DHODH is inhibited (Fig. 1). These results indicate that DHOA excretion would not be a useful measure of compliance in patients treated with LFM or BQR. However, the modified HPLC method reported enabled simultaneous separation of the first four intermediates in the de novo pyrimidine synthetic pathway – carbamoyl phosphate, carbamoyl aspartate, DHOA and OA. It thus may be of use also to identify putative genetic metabolic defects in humans involving DHODH, or earlier steps in the pathway. To date deficiency of UMP synthase (catalysing the last two steps, Fig. 1) is the only defect of pyrimidine de novo synthesis identified [21]. Since our HPLC method will separate OR, the intracellular breakdown product of OMP, as well (Fig. 1), all five intermediates in the pathway may be distinguished in any experimental situation requiring simultaneous separation of these components.

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