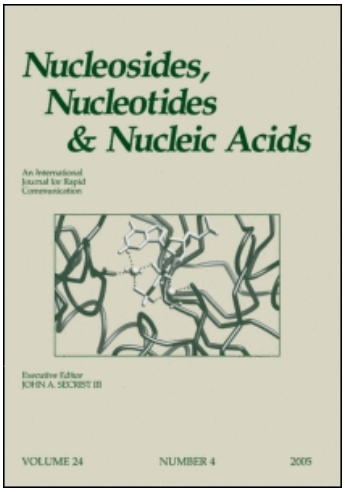

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 31 March 2001

To cite this Article Coussot, G. , Lefebvre-Tournier, I. , Hollecker, L. , Gosselin, G. , Imbach, J. -L. and Pompon, A.(2001) 'SIMULTANEOUS HPLC ANALYSIS OF HYDROPHOBIC PRODRUGS AND THEIR HYDROPHILIC METABOLITES IN BIOLOGICAL MEDIA WITHOUT SAMPLE PREPARATION. APPLICATION TO THE BIOTRANSFORMATION STUDIES OF PRONUCLEOTIDES DERIVED FROM 5-FLUOROURACIL', Nucleosides, Nucleotides and Nucleic Acids, 20: 4, 855 — 858
To link to this Article: DOI: 10.1081/NCN-100002445
URL: http://dx.doi.org/10.1081/NCN-100002445

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 20(4–7), 855–858 (2001)

SIMULTANEOUS HPLC ANALYSIS OF HYDROPHOBIC PRODRUGS AND THEIR HYDROPHILIC METABOLITES IN BIOLOGICAL MEDIA WITHOUT SAMPLE PREPARATION. APPLICATION TO THE BIOTRANSFORMATION STUDIES OF PRONUCLEOTIDES DERIVED FROM 5-FLUOROURACIL

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ABSTRACT

Improvements of an "on-line cleaning" HPLC method for analysis of biological samples are presented: (i) the use of cleaning precolumns filled with hydrophobic stationary phases instead of the hydrophilic ones previously used to eliminate the biological matrix; (ii) the combination in the mobile phase of anionic and cationic pairing reagents in order to retain on the precolumn all the metabolites, whatever their hydrophilicity and ionicity are. Such modifications allowed to study the biotransformation of prodrugs of 5-Fluorouracil, designed to act as antitumoral pronucleotides.

In order to study the behavior of oligonucleotides in various models of biological media (cell culture medium, sera, cell extracts), we introduced some years ago an "on-line cleaning" HPLC methodology (1). This method was adapted to the study of antiviral didesoxynucleotide prodrugs (pronucleotides) (2). Briefly,

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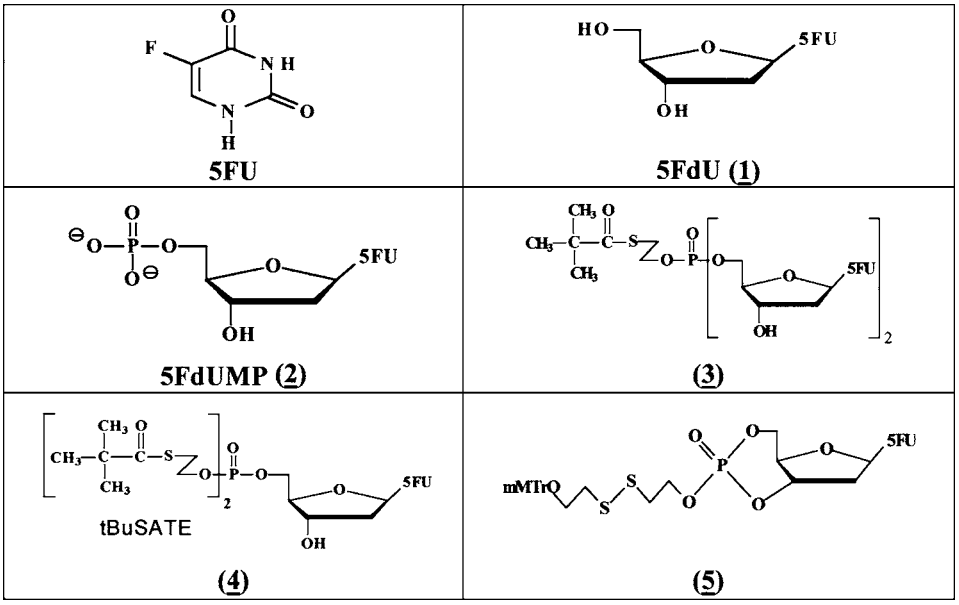

Figure.

the crude sample was injected in a special precolumn which was able to retain the analytes (pronucleotide and its metabolites), while the matrix (mainly proteins) was eliminated. Then, the trapped analytes were transferred by a switching technique in an analytical column, chromatographed and detected by UV and Mass spectrometers. This methodology has been successfully applied to several anti-HIV pronucleotides (2–5). The knowledge of the metabolic pathways and kinetic parameters could be related to the biological evaluation data, helping us to design new series of prodrugs.

In an attempt to extend the pronucleotide strategy to anticancer agents, some 5-Fluorouracil (5-FU) derivatives **3**–**5** (see Fig.) were synthesized. Unfortunately, our previously reported HPLC methodology (2–5) was not satisfactory to study the biotransformation of such compounds. Indeed, two very hydrophilic metabolites, the desoxynucleoside (5-FdU **1**) and the desoxynucleotide (5-FdUMP **2**), were not retained on the precolumn and were eliminated with proteins during the cleaning stage.

Our challenge was to find suitable stationary and mobile phases to eliminate the whole biological matrix, while retaining both hydrophobic and hydrophilic analytes during the cleaning stage. The stationary phase previously used consisted of porous C_{18} particles externally coated with an hydrophilic film of polymerized albumin. Proteins are not retained on this film and are quickly eliminated, while likely hydrophobic analytes are trapped inside the pores. We thought we would be able to eliminate proteins while retaining hydrophilic analytes when using, on the contrary, a very hydrophobic phase. From the twelve commercial precolumns

tested, such a very hydrophobic material with very low silanol activity was selected (*Symmetry Shield C_{18}, 20 × 3.9 mm, WATERS*). As the expected metabolites **1** and **2** were very hydrophilic entities, we decided to carried out assays with ion-pairing systems. Indeed, we were able to retain either the nucleotide **2** using an anion pairing reagent [tetrabutyl ammonium hydrogenosulfate, (Pic A-WATERS), 0.375 mM in water, pH 7.0] or the nucleoside **1** when we used a cation pairing reagent [heptanesulfonic acid (Pic B7-WATERS, 0.2 mM in water, pH 3.0]. Surprisingly, when further attempts were performed with a mixture of the two ion-pairing reagents (Pic A, 0.5 mM + Pic B7, 0.2 mM, pH 6.8) both **1** and **2** were retained as well as the parent prodrugs and the intermediate metabolites. To our knowledge, it is the first time that a double ion-pairing technique was used to extract simultaneously several metabolites from a biological matrix whatever their ionic charge and hydrophilicity. In our new conditions, the matrix was fully eliminated in less than 1 min (flow rate 1.0 mL/min). Then the precolumn was connected to the analytical column (*Hypersil BDS C_{18} 100 × 4.6 mm, THERMOQUEST*) and the trapped analytes were transferred using a triethylammonium acetate buffer (20 mM, pH 6.8) and chromatographed with a gradient of acetonitrile.

The reported improvements allowed us to study the behaviour of compounds **3**–**5** in human serum and CEM cell extracts. In cell extract, compound **3** decomposed ($t_{1/2} = 2.5$ h) into the corresponding 5′-5′ dinucleosidic phosphodiester derivative after the removal of the *S*-acyl-2-thioethyl protecting group (t-Butyl SATE). This first metabolite was very stable and neither derivatives **1** and **2** were observed. Concerning compound **4** in human serum, as expected the two SATE groups were slowly eliminated ($t_{1/2} = 9$ and 14 h, respectively). However, only traces of **2** were observed beside the formation of **1** and of an unknown metabolite. Similar results were obtained following incubation of **4** in cell extract, the SATE groups were quickly eliminated ($t_{1/2} = 2$ and 5.5 h, respectively), but we could not observe the nucleotide **2**. The determination of the decomposition pathways for compounds **3** and **4** showed that they were not able to deliver the nucleotide **2** in cell extract. These data might be the basis of an explanation of the lack or the low activity observed for these compounds in several thymidine kinase deficient (TK^-) cell lines (data not shown).

When the 3′,5′ cyclic (mMTr-DTE) phosphotriester derivative **5** was incubated in CEM cell extract, the protecting group was cleaved ($t_{1/2} = 4$ h). The resulting 3′,5′ cyclic phosphate derivative was detected and appeared to be stable after 3 days, therefore the nucleotide **2** was not obseved. Currently, we are trying to correlate the results of our decomposition studies with the antitumoral data obtained in cell culture experiments. However that may be, we have to keep in mind that: (i) cell extracts are approximate models of living cells, their preparation (lysing of the cells, centrifugation, freezing) can extensively modify the enzyme activities; (ii) if a metabolic pathway is observed in a cell extract, very likely it will exist in cells; (iii) if a metabolic pathway is not observed in cell extract, by no means one can extrapolate to cells. For instance, when a 3′,5′ cyclic phosphodiesterase was added together with **5** to the cell extract, the fast formation of the nucleotide **2** occurred.

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In conclusion, two improvements of our previous on-line HPLC cleaning method (1–5) allowed, for the first time, to study the biotransformation of potential prodrugs derived from 5-Fluorouracil. These modifications open wide possibilities to study the behaviour in various biological media of mixtures of hydrophobic derivatives and their hydrophilic metabolites whatever their ionic charge is.

ACKNOWLEDGMENTS

These investigations were supported by grants from the french "Fondation pour la Recherche Médicale" (FRM) and from the french "Ligue Nationale contre le Cancer". We thanks the Waters Company (Saint Quentin en Yvelines, France) for a generous gift of HPLC materials. We are indebted to Dr S. Peyrottes for critical review of the manuscript.

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