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Note

Separation of 2-fluoro-2-deoxyarabinofuranosylpyrimidine nucleosides by high-performance liquid chromatography

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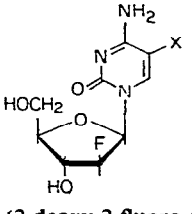
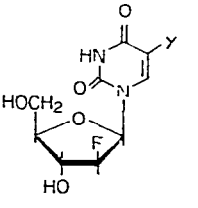
Recently, a series of 5-substituted 1-(2-deoxy-2-fluoroarabinofuranosyl)pyrimidines have been found to be antiviral agents[1]. Three of the more potent members of the series are 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC, 1 in Table I), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU, 4) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU, 5). These substances inhibit the replication of HSV-1 *in vitro* while having relatively little toxicity to host cells. Both FIAC and FMAU have high activity *in vivo*, with FMAU being the more potent against HSV-1.

TABLE I

STRUCTURE OF THE 2-FLUORO-2-DEOXYARABINOFURANOSYLPYRIMIDINE NUCLEOSIDES





R = 1-(2-deoxy-2-fluoro-β-D-arabinosyl).

Compound	*X*	*Compound*	*Y*
1 R-5-iodocytosine (FIAC)	I	4 R-5-iodouracil (FIAU)	I
2 R-5-Methylcytosine (FMAC)	CH_3	5 R-5-Methyluracil (FMAU)	CH_3
3 R-Cytosine (FAC)	H	6 R-Uracil (FAU)	H

FIAC is converted *in vivo* into FIAU by deamination. It is conceivable that FIAC could form FMAU by deamination, deiodination, and methylation. The last two metabolic steps have been demonstrated in the conversion of 5-iodo-2′-deoxyuridylate into thymidylate[2]. Since any viral activity attributed to FIAC *in vivo* could be at least in part due to its biotransformation into FIAU or FMAU or both,

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we were prompted to develop an analytical system that could detect the presence of any of these antiviral metabolites in tissue extracts. In developing the system and using it for tissue extracts, we found, surprisingly, radioactivity not only in peaks corresponding to FIAC, FIAU, and FMAU, but also to FAC and FAU[3,4]. Our analytical system had to then be able to separate all these compounds. We were also interested in whether the presence of a halogen in the sugar or base moiety caused a significant change in the retention times of nucleosides. This paper describes a method using a reversed-phase high-performance liquid chromatographic procedure with Partisil ODS-1 and ODS-3 for the resolution of these compounds.

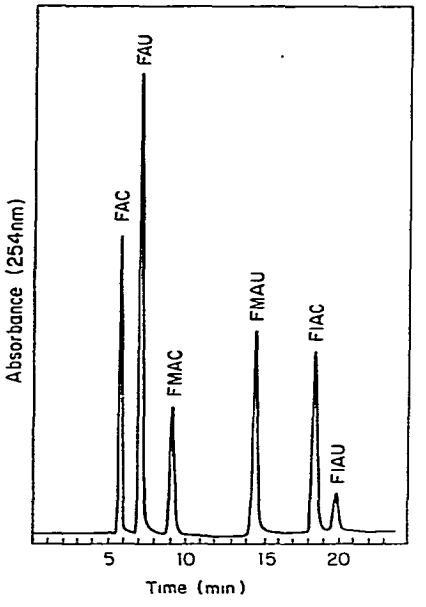
EXPERIMENTAL

A Spectra-Physics 8000 high-performance liquid chromatograph, complete with data system, was used for recording and integrating all peaks. Peaks were monitored at 254 nm with a Spectra-Physics 8300 fixed-wavelength detector. Partisil columns (5-μm ODS-1 and 10-μm ODS-3) were purchased from Whatman (Clifton, NJ, U.S.A.). Guard columns packed with Copell obtained from Whatman were used for all chromatographic runs. Samples were injected with a syringe into a 25-μl sample loop attached to a Valco (Houston, TX, U.S.A.) valve. Water and methanol, HPLC grade, were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Reagent grade sodium phosphate was from Mallinckrodt (St. Louis, MO, U.S.A.). The liquid scintillation solution hydrofluor was obtained from National Diagnostics (Sommerville, NJ, U.S.A.). The freon-amine was made by dissolving trichlorotrifluoroethane obtained from Fisher Scientific (Springfield, NJ, U.S.A.) in trioctylamine obtained from Aldrich (Metuchen, NJ, U.S.A.). Compounds 1–6 (see Table I) were gifts from Dr. J. J. Fox and co-workers (Sloan-Kettering Institute, New York, NY, U.S.A.). The marrow sample was obtained from Dr. T.-C. Chou (Sloan-Kettering Institute, New York, NY, U.S.A.). Amounts of 10–20 μmoles of each sample were injected onto the instrument for routine analysis.

RESULTS AND DISCUSSION

Table I lists the structures of six halogenated nucleosides that were studied. A successful separation of these compounds is shown in Fig. 1. This was obtained by eluting isocratically with 0.01 *M* sodium phosphate, pH 5.3, containing 4% methanol for 5 min, followed by isocratic elution with the same buffer in 20% methanol. The components eluted in the order of FAC, FAU, FMAC, FMAU, FIAC and FIAU. When elution was carried out isocratically with 4% methanol alone in 0.01 *M* phosphate, pH 5.3, or with a 0–30% methanol gradient in this buffer over a 30-min period, both FIAC and FIAU eluted as broad tailing peaks. Isocratic elution with higher amounts of methanol, such as 15 or 20%, failed to resolve FAC and FAU.

Although this system worked well for separating nanomolar mixtures of these compounds, when the amount of FIAC injected was increased to 0.05 mmoles or greater, FIAC and FIAU were not well resolved from each other even if the amount of FIAU co-injected was only several micromoles. When the buffer was changed to 0.02 *M* sodium phosphate, pH 3.0, FIAC and FIAU were well resolved for amounts up to 0.1 mmoles FIAC. (Higher amounts were not attempted.) This is shown in Fig. 2. With this change, the order of elution became FAC, FMAC, FAU, FMAU, FIAC and FIAU because FAU was retained longer at the lower pH.



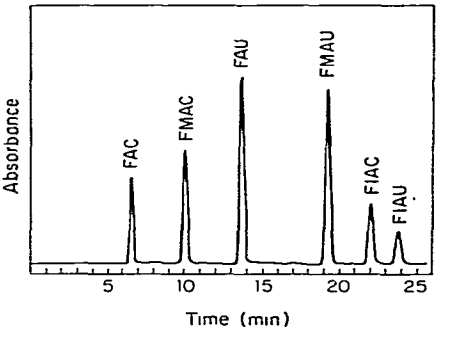


Fig. 1. Separation of 2-fluoro-2-deoxyarabinofuranosylpyrimidine nucleosides. Column 25 cm × 4.6 mm I.D. 10-μm Partisil ODS-1; solvent 0.01 *M* sodium phosphate, pH 5.3–methanol (96:4) for 4 min followed by 0.01 *M* sodium phosphate, pH 5.3–methanol (80:20); flow-rate 1 ml/min; temperature 40°C; detection, UV absorbance at 254 nm.

Fig. 2. As Fig. 1, except that the buffer is 0.02 *M* sodium phosphate, pH 3.0.

It appears from Figs. 1 and 2 that FIAC and FIAU have a strong affinity for the C_{18} resin. Under the conditions shown in Fig. 2, cytosine, 2′-deoxycytidine, 5-iodocytosine, 5-iodouridine, and 2′-deoxy-5-iodocytidine had retention times of 3.3, 4.4, 7.9, 12.1, and 18.0 minutes, respectively. (2′-Deoxy-5-iodocytidine and 2′-deoxy-5-iodouridine eluted at the same retention time.) It is apparent that the combination of the iodinated base and fluorinated sugar causes the long retention of FIAC and FIAU by the resin.

We observed that the back pressure of the 5-μm ODS-1 column increased with usage, particularly after a number of samples extracted from tissue had been applied. It was found that the 10-μm ODS-3 column gave almost as good a resolution as the 5-μm ODS-1 column with only 15–20% of the back pressure; it also had a considerably longer life-time.

The following results show the successful separation of FIAC and FIAU using the ODS-3 column. A sample of bone marrow which had been incubated with [^{14}C]FIAC for 45 min was obtained. The sample was homogenized in 10% perchloroacetic acid (PCA) to precipitate the DNA and proteins. The PCA was extracted with freon-amine[5]. The neutral supernatant was concentrated, centrifuged, and injected onto the chromatograph.

Fractions of 0.5 ml were collected manually into scintillation vials; 4.5 ml hydrofluor was added, and the vials were counted. The data are shown in Fig. 3. It can be seen that both the FIAC and FIAU radioactive peaks coincided with their

absorbance peaks. The percent deamination by the marrow cells, based on radioactivity, was 48%. This compared favorably to the 46% deamination value obtained by eluting part of the same sample through a Dowex 50 column and counting the eluent. This column had been shown to retain FIAC but not FIAU[6].

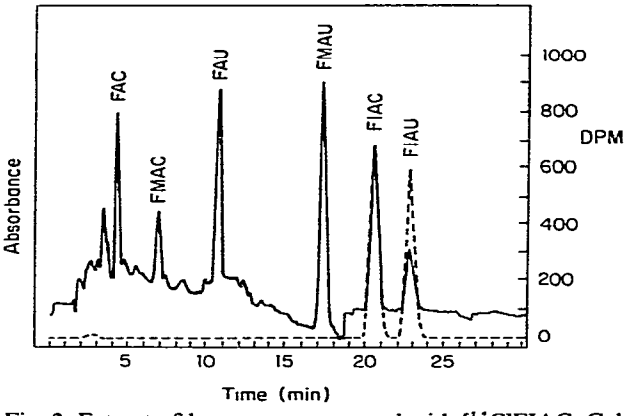


Fig. 3. Extract of bone marrow treated with [^{14}C]FIAC. Column 25 cm × 4.6 mm I.D., 10-μm Partisil ODS-3; other conditions as in Fig. 1.

Since we are studying the incorporation of these compounds into the acid-soluble fractions as well as into the DNA, we checked their separation in the presence of the four major deoxynucleosides. All the compounds were resolved, except that FAU partially overlapped the deoxyguanosine peak.

CONCLUSION

2-Fluoro-deoxyarabinofuranosylpyrimidine nucleosides can be separated efficiently by high-performance liquid chromatography using reversed-phase columns.

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