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PREPARATIVE AND ANALYTICAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS IN THE SYNTHESIS AND ANALYSIS OF DECOMPOSITION OF NITROSOUREA NUCLEOSIDES

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SUMMARY

Details are given for reversed-phase, adsorption, and aqueous ammonia-modified adsorption high-performance liquid chromatographic systems developed to separate 3′-chloroethylnitrosourea analogues of thymidine, 2′-deoxyuridine, and 5-fluoro-2′-deoxyuridine from their decomposition products and synthetic precursors. The effect of varying the substituent at the 3′- and 5-position on relative retention in each system is discussed. These systems are used to purify intermediates in the synthesis of these potent antineoplastic agents, and for the simultaneous analysis of the nitrosourea nucleosides and their breakdown products in kinetic studies of their decomposition. Application of these methods to the analysis of the kinetics of the breakdown of these compounds is demonstrated, with detection limits (signal-to-noise ratio = 2) in the 1–2 ng range.

INTRODUCTION

The anticancer activity both *in vitro* and *in vivo* of certain 2-haloethyl nitrosoureas is well established[1,2]. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), and 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea (MeCCNU) are in clinical use, but produce bone marrow toxicity. Attempts to ameliorate the toxic side effects of nitrosoureas while maintaining or improving antineplastic activity have led to the synthesis of a variety of analogues in which the carrier portion of the nitrosourea moiety has been altered[3–7]. This approach has produced two D-glucopyranose derivatives, streptozocin and chlorozotocin, which show reduced myelosuppression[8,9].

These data coupled with the availability of aminonucleosides in our laboratory[10–13] led to the synthesis of the nitrosourea nucleosides 3′-CTNU[14], 3′-CFdUNU, and 3′-CdUNU[15] (IV, VIII and XII, Table I). Each proved to have excellent antineoplastic activity, having an ED_{50} (L1210 cells) of 1.5, 2.5, and 12.0 μM respectively. In our research into the site and mode of action of these compounds, it became necessary to develop both preparative and analytical high-performance liquid chromatographic (HPLC) separations in order to prepare moderate quantities for bio-

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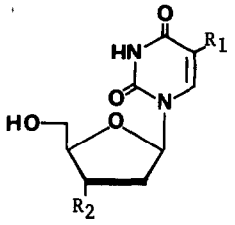
TABLE I

STRUCTURES OF THE COMPOUNDS STUDIED



Compound		*Abbreviation*	R_1	R_2
I	3'-Azido-3'-deoxythymidine	3'-N_3dThd	CH_3	N_3
II	3'-Amino-3'-deoxythymidine	3'-AdThd	CH_3	NH_2
III	3'-[3-(2-Chloroethylureido)]-3'-deoxythymidine	3'-UdThd	CH_3	$NHCONHCH_2CH_2Cl$
IV	3'-[3-(2-Chloroethyl)-3-nitrosoureido]-3'-deoxythymidine	3'-CTNU	CH_3	$NHCON(NO)CH_2CH_2Cl$
V	3'-Azido-2',3'-dideoxy-5-fluorouridine	3'-N_3FdUrd	F	N_3
VI	3'-Amino-2',3'-dideoxy-5-fluorouridine	3'-AFdUrd	F	NH_2
VII	3'-[3-(2-Chloroethylureido)]-2',3'-dideoxy-5-fluorouridine	3'-UFdUrd	F	$NHCONHCH_2CH_2Cl$
VIII	3'-[3-(2-Chloroethyl)-3-nitrosoureido]-2',3'-dideoxy-5-fluorouridine	3'-CFdUNU	F	$NHCON(NO)CH_2CH_2Cl$
IX	3'-Azido-2',3'-dideoxyuridine	3'-N_3dUrd	H	N_3
X	3'-Amino-2',3'-dideoxyuridine	3'-AdUrd	H	NH_2
XI	3'-[3-(2-Chloroethylureido)]-2',3'-dideoxyuridine	3'-UdUrd	H	$NHCONHCH_2CH_2Cl$
XII	3'-[3-(2-Chloroethyl)-3-nitrosoureido]-2',3'-dideoxyuridine	3'-CdUNU	H	$NHCON(NO)CH_2CH_2Cl$

chemical investigations and to study the kinetics of the decomposition of these reactive compounds.

In the past the decomposition of nitrosoureas has been followed by colorimetry[16], nitrogen evolution[17], thin-layer chromatography[18–20], chloride ion titration[16,20], gas chromatography–mass spectrometry (GC–MS)[21–23] and to a limited extent HPLC[23–26]. Of these methods, GC–MS and HPLC are by far the most sensitive and selective. In the case of the nitrosourea nucleosides, the non-volatility, strong UV absorbance, and the need for a preparative technique makes HPLC the method of choice over GC–MS.

Most of the published work concerning HPLC of nucleosides and nucleoside derivatives has involved the use of reversed-phase liquid chromatography (LC)[27–30]. Decomposition of nitrosoureas, however, is rapid in aqueous buffers; therefore, on-column degradation is a potential problem when employing reversed-phase LC. In recent years a number of papers reporting the successful use of underivatized silica

gel in the separation of nucleosides have appeared in the literature[31–34]. These separations were performed on underivatized silica gel employing mobile phases consisting of a water-immiscible organic solvent, an alcohol, and 0.4–3.0% of an aqueous solution of an ionizable substance. Ryba and Beranek[34] reported retention data for more than 50 pyrimidine and purine compounds on underivatized silica using dichloromethane–methanol–ammonium formate–formic acid solutions as eluents.

There are no previous reports comparing and evaluating HPLC methods for nitrosourea nucleosides. This paper reports the optimization of reversed-phase, adsorption, and aqueous ammonia-modified adsorption HPLC of nitrosourea nucleosides, their synthetic precursors, and their decomposition products. The methods are briefly compared and the suitability of each for particular applications discussed.

EXPERIMENTAL

Chemicals and related materials

HPLC-grade chloroform was obtained from Mallinckrodt (St. Louis, MO, U.S.A.) and HPLC-grade methanol was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Water was double-distilled prior to use. Ammonium hydroxide (assayed 28.9% as NH_3), sodium acetate, and acetic acid were purchased from J. T. Baker. Organic solvents were dried over Aldrich (Milwaukee, WI, U.S.A.) 3A molecular sieve for 24 h and filtered through Rainin (Woburn, MA, U.S.A.) nylon-66 filters. Aqueous mobile phases were filtered through Millipore HA filters, and all mobile phases were degassed prior to use. 3′-CTNU and 3′-UdThd were synthesized according to the procedure of Brubaker, Jr. and Prusoff[35]. All other 3′-analogues were generously provided by Dr. T.-S. Lin.

Chromatographic equipment

Two chromatographs were used. Gradient elution normal-phase HPLC analyses were conducted on a system consisting of a modified DuPont 830 liquid chromatograph in which the Haskel pump was replaced with an Altex 100 pump. Absorbance of the effluent was monitored at 254 nm and 280 nm using two Altex 153 UV detectors in tandem (Altex, Berkeley, CA, U.S.A.). A Gilson chromatographic system was used for reversed-phase and ammoniacal normal-phase gradient elution HPLC analyses. This system consisted of two Rainin Rabbit HP solvent delivery modules, a Gilson manometric module (Model 802B) and dynamic mixer (Model 811), and a Rheodyne sample injection valve (Model 7125). Absorbance of the effluent was monitored at 254 nm and 280 nm using two Altex 153 UV detectors, and recorded using a Kipp & Zonen chart recorder (Rainin). The chromatographic system was interfaced to and controlled by an Apple IIe computer.

Columns and chromatographic conditions

For reversed-phase separations a Whatman Partisil ODS-2 (25 × 0.46 cm I.D., 10 μm) column was used. Mobile phase A was 0.01 *M* KH_2PO_4 (pH 3.0)–methanol (95:5) and mobile phase B was 0.01 *M* KH_2PO_4 (pH 3.0)–methanol (55:45). The linear gradient used was 0–100% B in 30 min at a flow-rate of 1.75 ml/min at ambient temperature.

Normal-phase separations were conducted on a Whatman Partisil M9 (25 ×

0.9 cm I.D., 10 μm) column. Mobile phase A was methanol–chloroform (1:99) and mobile phase B was methanol–chloroform (9:91). Exponential (concave) gradients were employed for optimal separations. For the 3′-analogues of deoxyuridine a gradient of 5–95% B in 30 min with the instrument gradient selector set at $n=2$ was employed. 3′-Analogues of thymidine and 5-fluorodeoxyuridine were chromatographed using an exponential gradient of 15–90% B in 30 min. The normal phase separations were performed at a flow-rate of 3 ml/min and at ambient temperature.

Ammoniacal normal-phase separations were performed on a Spherisorb-Si (25 × 0.46 cm I.D., 5 μm) column. Mobile phase A was methanol–chloroform–ammonium hydroxide (1:99:0.01) and mobile phase B was methanol–chloroform–ammonium hydroxide (12:88:0.01). A two-stage linear gradient was employed, 5–12.5% B for 10 min followed by 12.5–100% B in 30 min. The flow-rate was 1.75 ml/min. Chromatography was conducted at ambient temperature.

Kinetics procedure

A 100 μ*M* stock solution of 3′-CTNU in anhydrous ethanol was prepared and the concentration measured by UV scan (Beckman Model 25 spectrophotometer). A disposable glass vial was charged with 1900 μl of 0.2 *M* sodium acetate–acetic acid buffer adjusted to pH 5.0. Temperature was maintained at 37°C in a WCLID Model 2156 water bath shaker (Warner-Chilcott Labs., Richmond, CA, U.S.A.). At this point 100 μl of the 3′-CTNU stock was pipetted into the vial, the solution vortexed for 5 sec, and the time recorded as $t=0$. The vial was sealed with parafilm.

Aliquots for analysis by the HPLC methods developed for this study were taken at $t=0$, 1, 2, 3, 4, 5, 8 and 24 h. For analysis on the reversed-phase system, 15-μl aliquots were injected without manipulation. For simultaneous analysis on normal phase, a 200-μl aliquot was first adsorbed onto an Extrelut QE disposable silica column (EM Science, Gibbstown, NJ, U.S.A.) to remove water and salts, followed by quantitative elution of the 3′-CTNU with methanol–chloroform (5:95) into a 10-ml volumetric flask. An aliquot (200–500 μl) was taken from the flask for injection into the normal-phase system.Standard curves of 3′-AdThd and 3′-CTNU were constructed by triplicate injections of standard solutions at four concentrations in the range of interest. The standards were prepared using the primary mobile phases to dissolve the compounds. All standard curves passed through the origin. For kinetic data concentration *vs.* time was measured for 3′-AdThd and 3′-CTNU by comparing peak height for these compounds against their respective standard curves. The kinetic data represents the mean of triplicate runs.

RESULTS AND DISCUSSION

The retention times of the nitrosourea nucleosides and related 3′- and 5-substituted nucleosides on the three chromatographic systems employed are listed in Table II.

Reversed phase

Within a given series of nucleoside 3′-analogues (R_1 constant), excellent resolution was obtained in the reversed-phase system. A representative chromatogram of the thymidine series is shown in Fig. 1. Baseline separation of the four compounds

TABLE II

RETENTION TIMES FOR PYRIMIDINE DERIVATIVES

Columns and mobile phase systems: see experimental. Retention time in minutes.

R_2	*Reversed phase*			*Adsorption (aqueous)*			*Adsorption (anhydrous)*		
	R_1			R_1			R_1		
	CH_3	F	U	CH_3	F	U	CH_3	F	U
NH_2	4.9	3.3	2.8	27.6	27.7	28.9	*	*	*
N_3	10.9	8.3	8.0	4.3	5.1	6.0	15.8	16.6	23.1
$NHCONHCH_2CH_2Cl$	20.8	17.0	16.4	16.6	17.9	18.0	29.0	30.5	35.0
$NHCON(NO)CH_2CH_2Cl$	27.6	24.5	23.4	6.9	8.4	8.7	17.8	18.9	24.3

* Compound retained on column.

within each series was achieved, with adjacent peaks being separated by a minimum of 5 min within 28 min. By changing the gradient profile, it is possible to expand or contract the separation of a given pair of adjacent peaks while maintaining the same overall time for separation. While the nitrosourea and its three synthetic precursors in each series is fully resolved, it may be necessary to make minor adjustments in the gradient profile to effect separation of the wide variety of breakdown products which may be produced differing only in the structure of the moiety at the 3'-position.

The structure–retention relationships observed by varying the substitution of the pyrimidine ring (R_2 constant) and on the sugar (R_1 constant) yield two very consistent elution patterns. Varying R_2 while holding R_1 constant produces an elution order of $-NH_2$ < $-N_3$ < $-NHCONHCH_2CH_2Cl$ <

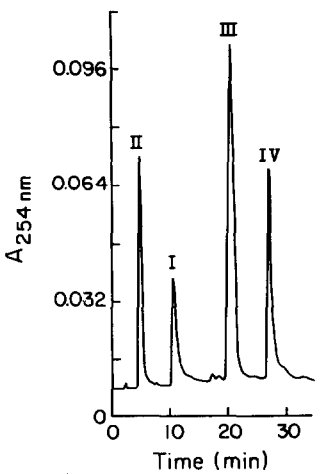


Fig. 1. Separation of 3'AdThd (II), 3'-N_3dThd (I), 3'-UdThd (III), and 3'-CTNU (IV). Column, Whatman Partisil ODS-2 (25 × 0.46 cm I.D., 10 μm); mobile phase A, 0.01 *M* KH_2PO_4 (pH 3.0)–methanol (95:5); mobile phase B, 0.01 *M* KH_2PO_4–methanol (55:45); linear gradient 0–100% B in 30 min, flow-rate 1.75 ml/min.

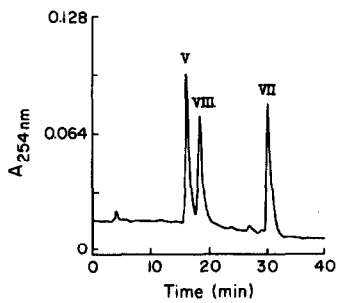
$-NHCON(NO)CH_2CH_2Cl$ in each case. As could be expected, the lipophilic chloroethyl moiety increases the retention relastive to the amino and azido analogues. These elution orders are in good agreement with and are qualitatively predicted by the molecular connectivity, χ, a topological index introduced by Kier *et al.*[36] and used by Karger *et al.*[37] as an estimator of retention in reversed-phase LC.

The second pattern, observed when varying R_1 with R_2 constant is $-H < -F < -CH_3$. As expected, the hydrophobic methyl group causes an appreciable increase in retention time relative to the corresponding -H or -F analogue, with the fluoro derivatives eluting at slightly longer retention times than the deoxyuridine derivatives.

Adsorption chromatography

While separations of nucleosides and their derivatives have traditionally been performed on reversed-phase LC, the few studies of the behavior of these compounds on adsorption chromatography have met with considerable success[30–34]. In addition, for our purposes the non-aqueous mobile phases generally employed may have certain advantages, since nitrosoureas are more stable under anhydrous conditions. Representative chromatograms of the anhydrous adsorption system and the aqueous ammonia-modified adsorption system are shown in Figs. 2 and 3. A number of interesting observations should be noted concerning elution order on reversed phase *vs.* adsorption and comparing overall retention in the two adsorption systems.

First, the elution order for R_2 constant is $-CH_3 < -F < -H$, the inverse of the order on reversed phase, as expected. The elution order found for varying R_2 provides an interesting illustration of the many factors influencing retention in ad-



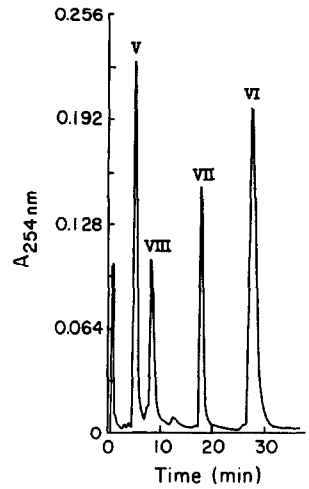


Fig. 2. Separation of 3'-N_3FdUrd (V), 3'-CFdUNU (VIII), and 3'UFdUrd (VII). Column, Whatman Partisil M9 (25 × 0.9 cm I.D., 10 μm); mobile phase A, methanol–chloroform (1:99); mobile phase B, methanol–chloroform (9:91); exponential (concave) gradient 15–90% B in 30 min, flow-rate 3.0 ml/min.

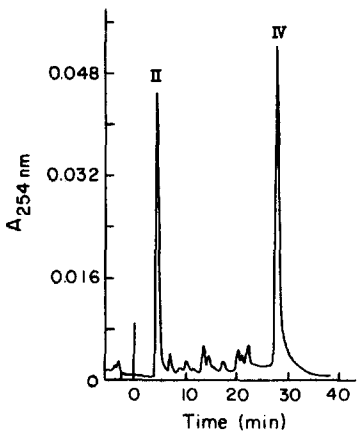
Fig. 3. Separation of 3'-N_3FdUrd (V), 3'-CFdUNU (VIII), 3'-UFdUrd (VII), and 3'-AFdUrd (VI). Column, Spherisorb-Si (25 × 0.46 cm I.D., 5 μm); mobile phase A, methanol–chloroform–ammonium hydroxide (1:99:0.01); mobile phase B, methanol–chloroform–ammonium hydroxide (12:88:0.01); two stage linear gradient, 5–12.5% B in 10 min followed by 12.5–100% B in 30 min, flow-rate 1.75 ml/min.

sorption chromatography. The general order is reversed with the exception of the azido compounds, which are displaced toward shorter relative retention times than that predicted by simply inverting the reversed-phase order. The affinity of the acidic surface of silica for weakly basic compounds is well known[38], routinely resulting in comparatively long retention times *versus* neutral or acidic solutes. In the present case this interaction results in the azido compounds becoming the earliest eluting peaks in each series, since the other compounds bear amino or ureido moieties at the 3′-position.

The two adsorption systems illustrate the effect of the addition of very small amounts of aqueous ammonia (0.01–0.015%, v/v). As illustrated in Fig. 2, 3 and Table II, anhydrous methanol–chloroform as eluent results in the amino compounds being retained on the column. A methanol concentration of over 50% is required to elute the amino compounds under anhydrous conditions, which produced chromatograms with unacceptable baseline shifts when running gradients from 1% up to 50% methanol in chloroform. Addition of as little as 0.01% aqueous ammonium hydroxide greatly reduces the retention of all the 3′-analogues, allowing even the amino compounds to be eluted within 29 min. The elution order remains the same as when using the anhydrous eluent, while the resolution of the nitrosourea and azido analogues is improved greatly. While adding water in varying amounts to the mobile phase in adsorption chromatography to reduce or adjust retention times is common practice[38], the use of aqueous ammonium salts[34] or ammonium hydroxide[33] has been less widely explored. At the low concentrations employed, we found no unusual problems with column degradation.

Applications

The utility of the HPLC systems developed here for the separation of 3′-analogues of thymidine, deoxyuridine, and 5-fluoro-2′,3′-dideoxyuridine has been demonstrated. Two aspects of the study of nitrosourea nucleosides to which we have recently applied these methods are in the synthesis of double-radiolabelled 3′-



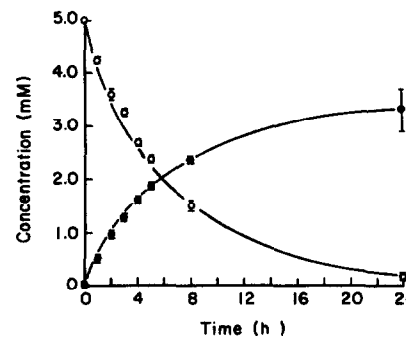


Fig. 4. Chromatographic profile of the decomposition of 3′-CTNU at 37°C in 0.2 *M* acetate buffer (pH 5.0), 5 h time point. Column and conditions as described for Fig. 1.

Fig. 5. Concentrations of 3′-CTNU (○) and 3′-AdThd (●) *vs.* time during incubation at 37°C in 0.2 *M* acetate buffer (pH 5.0). Initial concentration, 5 m*M* 3′-CTNU.

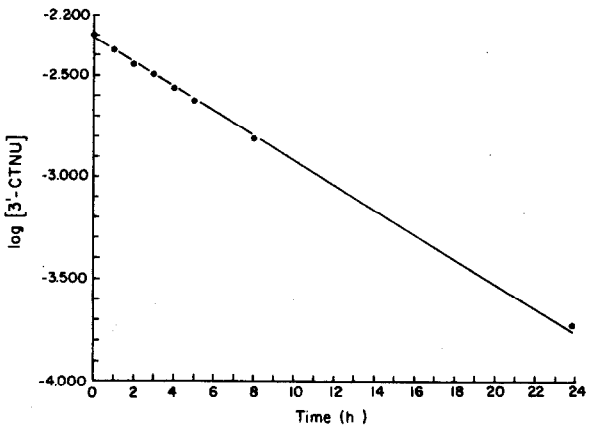


Fig. 6. Plot of log [3'-CTNU] *vs.* time indicating pseudo first-order kinetics for decomposition in 0.2 *M* acetate buffer (pH 5.0) at 37°C.

CTNU[35] for biochemical studies, and the mechanism of decomposition of 3'-CTNU[39]. In the former application, the use of the anhydrous methanol–chloroform adsorption system to purify 3'-UdThd before nitrosation coupled with a change from aqueous nitrosation to anhydrous nitrogen trioxide–chloroform improved the yield of 3'-CTNU from 43% to 90%[35].

The application of the reversed-phase system to the study of the kinetics of decomposition of nitrosourea nucleosides is illustrated for 3'-CTNU in Figs. 4, 5 and 6. The system allows simultaneous measurement of the rate of disappearance of 3'-CTNU and the formation of decomposition products. The predominant product, as identified by retention time and spectroscopic data is 3'-AdThd. One probable route to this compound is intermediate formation of the 3'-isocyanate followed by hydrolysis to the amine. Fisher *et al.*[40] previously have shown the production of 3'-AdThd in the decomposition of 3'-CTNU (phosphate-buffered saline, 37°C, pH 7.4), but the system used did not provide kinetic data for the rate of loss of 3'-CTNU. Further, the data was compromised by the chromatographic system (ion exchange) in which the high salt and elevated (58°C) temperature allowed breakdown of the nitrosourea during chromatography. By simultaneously injecting time points onto the adsorption system we found that the reversed-phase system used has no compromising effect on the kinetic data. The reversed-phase system is preferred over adsorption for kinetic studies because aqueous samples can be injected without manipulations. For injection on the adsorption systems, aqueous samples must first be passed through an Extrelut QE disposable silica column to remove water.

Fig. 5 shows the concentration *vs.* time dependence of 3'-CTNU and 3'-AdThd at 37°C in 0.2 *M* acetate buffer (pH 5.0). The points represent the mean of triplicate runs. HPLC analysis provided excellent reproducibility, with the average coefficient of variation for a set of concentration values at a given time point being 1.9%. A plot of log (3'-CTNU) *vs.* time (Fig. 6) obtained by least squares linear regression analysis showed excellent linearity ($r = 0.9995$), indicating pseudo first-order kinetics ($k = 3.76 \cdot 10^{-5}$ sec^{-1}, $t_{\frac{1}{2}} = 307$ min). Applications of these methods to the analysis of plasma and urine for pharmacokinetic studies will be published separately.

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