Journal of Chromatography A, 657 (1993) 208–212
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 555

Short Communication

Stability study of 2′-deoxyuridine by liquid chromatography

A. Van Schepdael*, E. Macken, R. Busson, G. Janssen, P. Herdewijn, E. Roets and J. Hoogmartens

Laboratorium voor Farmaceutische Chemie, Instituut voor Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, Van Evenstraat 4, B-3000 Leuven (Belgium)

(Received July 16th, 1993)

ABSTRACT

Previous stability studies on 2′-deoxyuridine (dUrd) made use of UV spectrophotometry. However, this technique does not allow dUrd to be determined separately from its anomer and pentopyranosyl isomers formed during acid degradation. The anomerization and isomerization have been monitored by TLC before, but the separation between dUrd and its α-anomer was not optimal. Therefore, the aim of this study was to determine the rate constants of the degradation of dUrd, using a recently developed liquid chromatographic method. Rate constants of degradation of dUrd were determined at several pH values in the range 1–12. Studies at different temperatures showed that the Arrhenius equation is applicable at pH 1 and 7. To elucidate further the mechanism of hydrolysis in acidic media, some degradation tests were performed at pH 1 on the α-anomer and pentopyranosyl isomers of dUrd.

INTRODUCTION

The stability of dUrd has been investigated mainly by UV spectrophotometry but this method has the disdvantage that the interpretation of the spectral data is hampered by the presence of uracil, as the main degradation product, and of a chromophore formed from the 2-deoxyribose part of the nucleoside molecule in acidic media. This chromophore can be eliminated, however, by working in alkaline media [1], and dUrd can be determined separately from uracil by measuring at two different wavelengths. The following pH ranges have been covered in stability studies: H_0 (Hammett acidity function) −0.92 to −6.47 [2], pH 0.03–0.61 [3] and pH 1.9–6.5 [4]. Later studies have shown that dUrd also anomerizes and isomerizes in strongly acidic media [5]. In this case thin-layer chromatography with radioactive scintillation counting was applied to resolve and determine all compounds formed [6]. We were interested in the stability of dUrd in the frame of a comparative liquid chromatographic stability study of 5-halogenated 2′-deoxyuridines. Therefore, we tried to determine dUrd selectively using liquid chromatography (LC) with the more convenient UV detection mode.

* Corresponding author.

0021-9673/93/$06.00

EXPERIMENTAL

2'-Deoxyuridine and 2-deoxy-D-ribose were purchased from Janssen Chimica (Beerse, Belgium). 1-(2-Deoxy-α-D-*erythro*-pentofuranosyl)-uracil (α-F) was synthesized according to a previously published procedure [7] and the syntheses of 1-(2-deoxy-α-D-*erythro*-pentopyranosyl)uracil (α-P) and 1-(2-deoxy-β-D-*erythro*-pentopyranosyl)uracil (β-P) have been described elsewhere [8]. All reagents were of analytical-reagent grade (Merck, Darmstadt, Germany) and water was distilled twice before use. Tetrahydrofuran was purified by distillation in the presence of iron(II) sulphate and was stored at 5°C in the dark. Stability studies were performed in the pH range 1.09–12.23; Glycine · HCl (0.1 *M*) was used for pH 1.09 and 0.1 *M* phosphate buffer for the other pH values. The ionic strength of all the buffers used was adjusted to 0.4 with KCl. pH was measured at room temperature with a Consort (Turnhout, Belgium) P514 pH meter using a Schott (Mainz, Germany) pH electrode. Samples were incubated at various temperatures in a Memmert (Schwabach, Germany) oven. The reaction was quenched at appropriate intervals by addition of a neutralizing KOH or HCl solution and freezing. All experiments were performed in duplicate.

Samples were analysed as a series by LC, using a Hypersil C_{18} 5-μm column (250 mm × 4.6 mm I.D.) with tetrahydrofuran–0.2 *M* phosphate buffer (pH 4.0)–water (0.1:5:94.9, v/v/v) as the mobile phase. The chromatographic equipment consisted of a Milton Roy mini-pump (Laboratory Data Control, Riviera Beach, FL, USA), used at a flow-rate of 1 ml/min, a Marathon injector (Spark Holland, Emmen, Netherlands) with a 20-μl loop, a Waters (Milford, MA, USA) Model 440 detector set at 254 nm and a Hewlett-Packard (Avondale, PA, USA) Model 3396 A integrator. The column temperature was maintained at 10°C using a jacket connected to a thermostat (Julabo, Seelbach, Germany). The usual starting concentration of dUrd in the LC samples was approximately 10^{-5} *M*. A Waters Model 990 photodiode array detector was used to record the on-line UV spectra.

Merck precoated silica gel F_{254} plates were used for TLC with CH_3CN–$CHCl_3$ (19:81, v/v) as the mobile phase. Column chromatography was performed on silica gel (Merck, 0.040–0.063 nm, 35 × 5.5 cm I.D.) with $CHCl_3$–CH_3CN (95:5, v/v) as the eluent. ^{13}C and ^{1}H NMR spectra were run, at the operating temperature, on a JEOL FX90Q spectrometer in 5-mm tubes, using deuterochloroform as solvent. Mass spectra were recorded on a Kratos Concept 1H mass spectrometer.

RESULTS AND DISCUSSION

The LC system was developed previously with a test mixture of dUrd, α-F, α-P and β-P in water [9]; a typical chromatogram is shown in Fig. 1. This system was found to be valid for quantification because a linear response of the detector was obtained in the kinetic working range. During kinetic studies, however, problems of peak splitting were encountered, probably owing to the very low percentage of organic modifier present in the mobile phase. It is believed that lipophilic components of the sample were retained on the stationary phase and

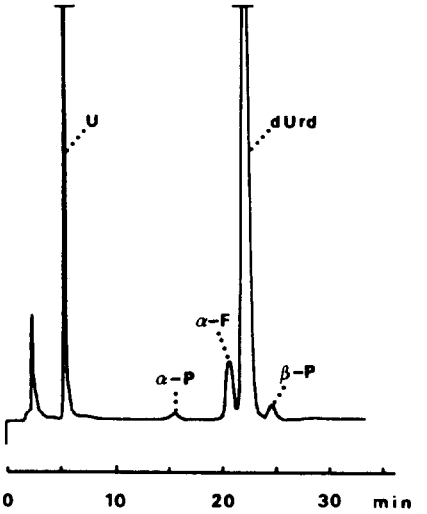

Fig. 1. Chromatogram of a sample of 2'-deoxyuridine degraded at pH 1.09 and 381 K for 4 h. Column, Hypersil C_{18}, 5 μm (250 mm × 4.6 mm I.D.); mobile phase, tetrahydrofuran–0.2 *M* potassium phosphate buffer (pH 4.0)–water (0.1:5:94.9, v/v/v); flow-rate, 1.0 ml/min; temperature, 10°C; detection, UV at 254 nm. U = Uracil.

shielded off the active silanol groups on its surface. Washing with non-polar solvents such as dichloromethane or with acidic polar mobile phases such as acetone–1 *M* perchloric acid–water (50:5:45, v/v/v) regenerated the column to its original state. To prevent these problems we looked for other types of stationary phases on which dUrd would be retained more strongly so that it would be possible to use larger amounts of organic modifier. The following columns were tested but did not improve the method: LiChrosorb diol (10 μm), RSil CN (10 μm) and RSil C_{18} HL (10 μm).

An acid degradation product of 2-deoxyribose is certainly one of the compounds retained on the column with this system, because it is seen in samples in kinetic studies, when analysed under the conditions applied previously for 5-halogenated 2′-deoxyuridines [10]. Chromatography on a Spherisorb ODS-1 (10 μm) column with methanol–0.2 *M* phosphate buffer (pH 5.0)–water (0.1:5:94.9, v/v/v) indeed revealed a substance which was identical (diode array detector; $\lambda_{max} = 262$ nm) with the compound formed through acid degradation of 2-deoxyribose under conditions described previously [1] and which differed from 2-furaldehyde or the acid degradation compounds described by Rice and Fishbein [11]. This compound was isolated by extraction of a degradation mixture of 2-deoxyribose (6 g in 500 ml of 1.2 *M* HCl, 80°C, 3 h) with chloroform. The extract was concentrated and purified by silica gel column chromatography. From mass spectrometry [electron impact ionization, *m/z* 98 ($M^{+\bullet}$), and isobutane chemical ionization, *m/z* 99 (MH^+)] and with the aid of ^1H NMR [δ 2.24 (d, $J = 0.8$ Hz, Me), 4.49 (AB, CH_2) and 5.49 ppm (q, $J = 0.8$ Hz, CH=)] and ^{13}C NMR [δ 16.6 (Me), 75.3 (CH_2O–), 104.7 (*HC*=C–O), 191.3 (=C–O–) and 202.5 ppm (conj. CO)], the compound was identified as 5-methyl-2,3-dihydrofuran-3-one, consistent with earlier results [12].

The degradation reaction of dUrd displayed first-order kinetics over the whole pH range studied. At pH 1.09 and 12.23 not only uracil was formed but also α-F, α-P and β-P, while at all other pH values only uracil was found as the degradation product. The identity of the chromatographic peaks was confirmed by comparison of retention times and by comparison of the UV spectra recorded by diode-array detection [λ_{max}(uracil) = 259 nm, λ_{max}(α-F) = 264 nm, λ_{max}(dUrd) = 262 nm, λ_{max}(α-P) = 260 nm and λ_{max}(β-P) = 260 nm]. Mass balance calculations performed on samples degraded at pH 1.09 and 7.00 fitted within a 4% deviation so that it was concluded that no major compounds were formed other than those described above. As no reference substances with known content were available for α-F, α-P and β-P, these substances were expressed as dUrd in the calculations. At pH 8.91 and 12.23, a decrease in mass occurred, probably because the pyrimidine ring opened in alkali, as has been described for uridine [13].

The hydrolysis of dUrd at various pH values and at 101°C is summarized in Table I. The ionic strength of all buffers was kept at 0.4 with KCl because of its influence on the reaction rate [2]. As the concentration of the phosphate buffer did not influence the degradation at pH 2.00 (see the data in Table I), and as previous investigators did not find buffer catalysis [4], all pH results were assessed together to determine different zones of catalysis. A previously described zone

TABLE I

OBSERVED RATE CONSTANTS FOR THE HYDROLYSIS OF dUrd AT 101°C AS A FUNCTION OF pH

0.1 *M* Glycine·HCl was used for pH 1.09 and 0.1 *M* phosphate buffer for the remaining pH studies. All buffers were of ionic strength 0.4. *N* = Total number of chromatograms; *x* = number of points on the time axis; *y* = number of independent experiments; *z* = number of half-lives during which tested.

pH	k (h^{-1})	*N*	*x*	*y*	*z*
1.09	0.060 ± 0.002	16	4	2	4
2.00	0.0207 ± 0.0005	35	9	2	4
	0.0188 ± 0.0005[a]	26	8	2	2
	0.0185 ± 0.0005[b]	28	8	2	2
2.95	0.0173 ± 0.0004	12	6	1	3
3.81	0.0159 ± 0.0003	28	8	2	3
4.57	0.020 ± 0.003	21	6	2	4
5.85	0.0219 ± 0.0007	16	8	1	4
7.00	0.025 ± 0.002	22	6	2	5
8.91	0.0086 ± 0.0001	13	7	1	3
12.23	0.023 ± 0.001	21	5	2	3

[a] 0.2 *M* phosphate buffer.
[b] 0.3 *M* phosphate buffer.

of specific acid catalysis at pH <0.7 [3] is followed by a zone of slope −0.5, which is probably a transition between the specific acid catalysis zone and the solvent catalysis zone in the pH range 3–7, where the overall slope is approximately zero. The slowest degradation was seen at pH 8.91. Previous workers had shown that the rate of hydrolysis falls off in the pH range 9–11 [4]. Our data at pH 12.23 show that k again rises at higher pH values. The degradation rate constants at pH 7.00 in 0.1 *M* phosphate buffer at 95°C, obtained with the UV spectrophotometric and the liquid chromatographic method, show a close resemblance, namely 0.011 h^{-1} (UV) and 0.012 h^{-1} (LC value calculated using the experimentally determined Arrhenius relationships below).

Fig. 2 depicts the possible mechanism of degradation at pH 1.09. The small amounts of anomer and isomers present could act as intermediates in the degradation of dUrd to uracil or could simply be side products of the degradation, not degrading to any significant extent to uracil. To elucidate this mechanism further, the anomer and isomers of dUrd were degraded under exactly the same conditions as dUrd itself and the observed degradation rate constants are given in Table II.

First it is interesting to note that α-F degrades 1.3 times faster than dUrd at pH 1.09, which is comparable to the factor of 1.4 found for 2'-deoxyadenosine and its α-anomer [14]. York [14] suggested that a reverse anomeric effect together with a steric strain from the *cis*-3'-OH was the cause of the decreased stability of the α- relative to the β-anomer. The presence of a ribopyranosyl sugar in all cases stabilizes the compound since α-F is 12.8 times more labile than α-P and dUrd is 3.2 times more labile than β-P. This is also consistent with the fact that 1-(2-deoxy-α-D-*erythro*-pentofuranosyl)adenine is 29.8 times more labile than 1-(2-deoxy-α-D-*erythro*-pentopyranosyl)adenine [14] and that the adenine and uracil nucleosides of ribo- and glucopyranose are hydrolysed more slowly than those of ribofuranose [15].

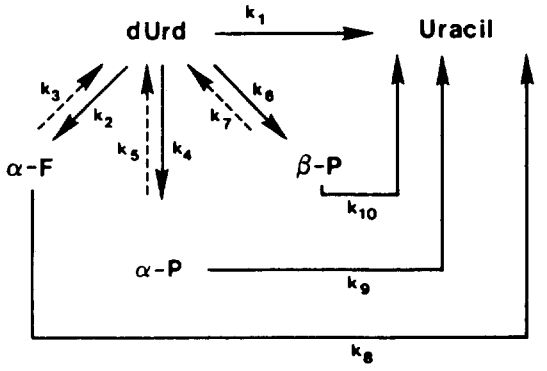

Fig. 2. Possible mechanism of degradation at pH 1.09.

TABLE II

OBSERVED RATE CONSTANTS OF DEGRADATION OF α-F, α-P, β-P AND dUrd AT pH 1.09 AND 101°C IN 0.1 *M* GLYCINE · HCl BUFFER ($\mu = 0.4$)

Compound	k (h^{-1})
α-F	0.077 ± 0.001
α-P	0.0060 ± 0.0002
β-P	0.0191 ± 0.0003
dUrd	0.060 ± 0.002

Further, during this experiment no dUrd could be detected in degradation samples of α-F, α-P or β-P, so that the equilibrium reactions in Fig. 2 can be neglected and k_3, k_5 and k_7 approximate to zero. It can also be concluded that k_8, k_9 and k_{10} are equal to the degradation rate constants observed for α-F, α-P and β-P, respectively. As the α-pyranosyl and β-pyranosyl forms degrade more slowly than dUrd and the α-furanosyl form more quickly, we tend to conclude that dUrd degrades to uracil either directly or mainly through its α-anomer. It should be possible to determine the contributions of k_1, k_2, k_4 and k_6 to the overall degradation rate constant of dUrd if standards of the intermediates were available. In that case a simulation of the experimentally obtained concentration profiles using a multiparameter computer program might be performed [16]. To achieve this, however, α-F, α-P and β-P standards of known purity must be synthesized.

The formation of the anomer and ring isomers of dUrd indicated that two pathways are followed in the degradation of dUrd: on the one hand a direct rupture of the N-glycosidic bond and on the other the formation of a Schiff base intermediate in which the opened sugar ring can recyclize in different ways, thus forming the

anomer or ring isomers [5]. As the percentage of recyclization of the Schiff base intermediate towards a five- or a six-membered sugar ring is not known, it is difficult to distinguish between the two pathways.

Table III gives the results of experiments performed at different temperatures. The following Arrhenius relationships were obtained:

pH 1.09: $\log k = 14.6 - 5934(1/T)$ $r = 0.9998$
pH 7.00: $\log k = 12.7 - 5371(1/T)$ $r = 0.9977$

Activation energies determined from the respective slopes were 26.9 and 24.6 kcal/mol (1 kcal = 4.184 kJ). These in turn yielded entropy of activation values of −11 e.u. at pH 1.09 and −19 e.u. at pH 7.00 and 75°C. The latter large negative value deviates substantially from the value of 3.5 e.u. previously determined at 75°C and pH 6.5 [4].

TABLE III

OBSERVED RATE CONSTANTS FOR THE HYDROLYSIS OF dUrd AT pH 1.09 (0.1 *M* GLYCINE · HCl, $\mu = 0.4$) AND pH 7.00 (0.1 *M* PHOSPHATE BUFFER, $\mu = 0.4$) AS A FUNCTION OF TEMPERATURE

N = Total number of chromatograms; *x* = number of points on the time axis; *y* = number of independent experiments; *z* = number of half-lives during which tested.

pH	*T* (K)	k (h^{-1})	*N*	*x*	*y*	*z*
1.09	348	0.0038 ± 0.0002	10	5	1	<1
	374	0.060 ± 0.002	16	4	2	4
	381	0.119 ± 0.005	27	7	2	5
	387	0.191 ± 0.003	24	6	2	6
7.00	338	0.00062 ± 0.00006	12	6	1	<1
	354	0.0029 ± 0.0002	30	6	2	<1
	374	0.025 ± 0.002	22	6	2	5
	387	0.056 ± 0.001	23	6	2	6

ACKNOWLEDGEMENTS

A.V.S. is a Senior Research Assistant of the Belgian National Fund for Scientific Research. The authors thank C. Vinckier (Afdeling Anorganische en Analytische Scheikunde, Katholieke Universiteit Leuven, Leuven, Belgium) for instructive discussions. A. Decoux and I. Quintens are acknowledged for secretarial help.

REFERENCES

1 J.K. Seydel and E.R. Garrett, *Anal. Chem.*, 37 (1965) 271.
2 R. Shapiro and M. Danzig, *Biochemistry*, 11 (1972) 23.
3 E.R. Garrett, J.K. Seydel and A.J. Sharpen, *J. Org. Chem.*, 31 (1966) 2219.
4 R. Shapiro and S. Kang, *Biochemistry*, 8 (1969) 1806.
5 J. Cadet and R. Téoule, *J. Am. Chem. Soc.*, 96 (1974) 6517.
6 J. Cadet and R. Téoule, *J. Chromatogr.*, 76 (1973) 407.
7 H. Aoyama, *Bull. Chem. Soc. Jpn.*, 60 (1987) 2073.
8 P. Herdewijn, A. Van Aerschot, R. Busson, P. Claes and E. De Clercq, *Nucleosides Nucleotides*, 10 (1991) 1525.
9 A. Van Schepdael, A. Van Aeroschot, P. Herdewijn, E. Roets and J. Hoogmartens, *Chromatographia*, 33 (1992) 571.
10 A. Van Schepdael, N. Ossembe, P. Herdewijn, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.*, 11 (1993) 345.
11 F.A.H. Rice and L. Fishbein, *J. Am. Chem. Soc.*, 78 (1956) 1005.
12 J.K. Seydel, E.R. Garrett, W. Diller and K.-J. Schaper, *J. Pharm. Sci.*, 56 (1967) 858.
13 A.S. Jones, A.M. Mian and R.T. Walker, *J. Chem. Soc. C*, (1966) 1784.
14 J.L. York, *J. Org. Chem.*, 46 (1981) 2171.
15 F. Micheel and A. Heesing, *Chem. Ber.*, 94 (1961) 1814.
16 C. Vinckier, R. Hauchecorne, Th. Cachet, G. Van den Mooter and J. Hoogmartens, *Int. J. Pharm.*, 55 (1989) 67.