concerned with influences of various solutes and vehicles on the physical states of cornified epithelial tissues. The presence of a natural hydrating factor which can be removed by the ether-water treatment appears to be clearly established by these studies. It is further evident that very dilute aqueous solutions of various salts and nonelectrolytes have pronounced effects on the configuration of the protein structure.

Some of these observations may be pertinent in formulation of skin preparations. Although the astringency of aluminum ion is well known, observation of the effect of sodium, potassium, lithium, and magnesium ion in producing relaxation of stressed tissue, even in dilute solutions, appears to be new. This behavior seems to be in opposition to the dehydrating effect normally produced by the presence of solutes in water.

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Procedure for Assay and Stability Determination of Idoxuridine

By CHARLES A. SIMPSON and ALFRED F. ZAPPALA

In acid solution, idoxuridine (5-iodo-2′-deoxyuridine) is decomposed by light to form 2′-deoxyuridine and material whose U.V. absorption has been destroyed. Idoxuridine is hydrolyzed by heat to form 5-iodouracil and 2-deoxyribose in nearly quantitative yield; a small amount of 2′-deoxyuridine is also formed. A procedure for assay of these mixtures is given. The method involves a chromatographic separation on a partition column with a standing phase of 0.1 *N* HCl and a moving phase of chloroform plus *n*-butanol (5 : 1 by volume). That portion of the eluate containing the idoxuridine is analyzed by its U.V. absorption. Data are presented to show the accuracy and precision of the method.

IDOXURIDINE (5-iodo-2′-deoxyuridine) was first synthesized in 1959 by Prusoff (1). Following its synthesis, several papers were published in connection with its effect on transplantable neoplasms (1–3). In addition, it was studied in its role as a bacterial inhibitor (1, 4). More recently, Kaufman has shown that it is an agent of proven value in treatment of herpetic keratitis in man (5). This has been hailed as the first chemotherapeutic agent effective against a specific virus. Because of this growing biochemical and medical interest in idoxuridine, we undertook to study its decomposition and find a method for stability determination. This report describes the results of that effort.

EXPERIMENTAL

Study of Light-Initiated Decomposition.—Unbuffered aqueous solutions of idoxuridine (1 mg./ml., pH = 5–6) were placed in quartz containers and irradiated with a mercury vapor arc lamp. Figure 1 shows that the U.V. absorption spectra of these solutions decrease in intensity with an increase in time of exposure. The nature of this decomposition product was not studied, since it appeared to offer no interference with an ultraviolet method of determination. Moreover, the photolytic decomposition of related compounds such as uridine and uracil has been studied by Sinsheimer and Hastings and many others (6). These studies showed that the product formed is the 4-hydroxyhydro derivative.

Another considerably less severe irradiation experiment was conducted with a Sylvania type RS sunlamp, where the spectrum includes portions of the I.R. and visible as well as the U.V. ranges. As previously, the solutions irradiated were unbuffered and aqueous (1 mg./ml., pH = 5–6). After 3 months of exposure, the solution was spotted on paper along with a known solution of some suspected decomposition products, and a chromatogram was developed in a butanol–3 *N* ammonia system. The results of this study are shown in Table I.

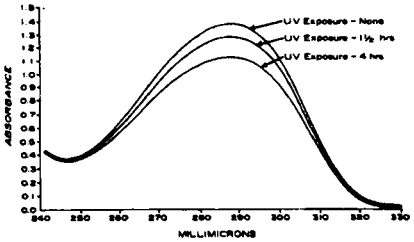

Fig. 1.—U.V. absorption spectra of aqueous idoxuridine exposed to U.V. light.

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The authors thank Mr. Alex Post of our laboratories for his paper chromatographic work which assisted greatly in this investigation. Recognition is also given to Dr. Herbert Winicov, under whose direction the pure idoxuridine standard was prepared.

TABLE I.—COMPARISON OF R_f VALUES OBTAINED FOR SOME POSSIBLE DECOMPOSITION PRODUCTS AND A SUNLAMP-DECOMPOSED AQUEOUS SOLUTION OF IDOXURIDINE

Material	R_f Values: Known Soln.	R_f Values: Sunlamp Decomposed Soln.
Unknown	...	0.00 (Significant)
2'-Deoxyuridine	0.23	0.22 (Significant)
Idoxuridine	0.31	0.31 (Large)
2-Deoxyribose	0.37	0.37 (Small)
5-Iodouracil	0.50	0.47 (Small)

The formation of a small amount of deoxyribose and iodouracil was expected, since the writers showed in an earlier note that these are the principal thermal decomposition products (7). The major breakdown is to deoxyuridine and material giving no absorbance or fluorescence in U.V. light, yet still giving the characteristic deoxyribose pink color with cysteine and sulfuric acid. As before, this may indicate the hydration of the 4,5 double bond or the destruction of the ring.

Study of Heat-Initiated Decomposition.—In the solid state, idoxuridine shows considerable stability toward heat. After storage for 9 months at 60°, its paper chromatogram and U.V. assay were unchanged from the original. This is not the case with aqueous solutions, however. Unbuffered aqueous solutions of idoxuridine (1 mg./ml., pH = 5–6) were stored for a few months at temperatures ranging from room temperature to 45°. They were then spotted on paper along with a solution of suspected breakdown products, and chromatograms were developed in a butanol–3 *N* ammonia system. The results of this study are shown in Table II, which shows that the initial breakdown is to deoxyuridine. However, its rate of formation is relatively low; the main decomposition results in the nearly quantitative formation of iodouracil and deoxyribose. This rise in iodouracil content, coincidental with the decline in idoxuridine, was determined using the chromatographic procedure to be described later. These data are shown in Table III.

Since uracil is not well separated in the paper system described above, those same solutions were also chromatographed in a 2-methyl-3-butyn-2-ol, pH 8 buffer system. No uracil was found in these samples. Subsequent studies have shown that uracil can only be found in badly decomposed solutions (50% or more degraded). In fact, uracil was the only pyrimidine compound still present after treatment of an aqueous unbuffered solution for 17 hours at 100°.

Some decomposition studies were also conducted at pH's as high as 10. This aspect was not investigated to a great degree, except to observe that the ionized form of idoxuridine (pKa ~ 8) is considerably less stable than the acid form.

Method Development.—The determination of idoxuridine can be accomplished in a number of ways. Its deoxyribose moiety will react with cysteine in 75% v/v H_2SO_4 to form a pink color (8, 9). A direct method of assay for the pyrimidine base is based upon its ability to absorb four equivalents of iodine from basic solution (10, 11). There is a colorimetric method which uses the ability of the base to reduce arsenotungstic acid (12). Finally, idoxuridine can be determined from its U.V. absorption spectrum ($\epsilon_{max.}$ = 7763).

Unfortunately, in each of these methods one or more of the breakdown products interferes. The complex nature of this interference is illustrated by the U.V. absorption spectra in Fig. 2. Consequently, it was desirable to devise a relatively simple but versatile system of separation, one which would be capable of the stability determination of idoxuridine in research systems where it would be mixed with a variety of drug substances or where the pH would be varied through a considerable part of the aqueous range. After separation, any suitable property could be selected for measurement. The separation technique chosen was liquid-liquid partition chromatography on a Celite 545 support; the property chosen for measurement was U.V. absorption.

Partition Column.—Because the great majority of situations under study involved aqueous solutions,

TABLE II.—COMPARISON OF R_f VALUES OBTAINED FOR SOME POSSIBLE DECOMPOSITION PRODUCTS AND HEAT-DECOMPOSED AQUEOUS SOLUTIONS OF IDOXURIDINE

Material	R_f Values: Known Soln.	R_f Values: 3 Mo. at Room Temp.	R_f Values: 3 Mo. at 45°
2'-Deoxyridine	0.23	0.24 (Trace)	0.23 (Trace)
Uracil	0.28	...	...
Idoxuridine	0.31	0.32 (Large)	0.31 (Large)
2-Deoxyribose	0.37	...	0.39 (Significant)
5-Iodouracil	0.50	...	0.48 (Significant)
Idoxuridine assay, % of original		98	69

TABLE III.—MATERIAL BALANCE OF IDOXURIDINE AND 5-IODOURACIL IN THERMAL DEGRADATION

Sample	Storage Condition	Mole % of Original: Idoxuridine	Mole % of Original: 5-Iodouracil	Mole % of Original: Total
Aqueous, 1 mg./ml., pH 5.0, buffered	1 wk. at 60°	79	21	100
Aqueous, 1 mg./ml., pH 6.2, buffered	1 wk. at 60°	76	22	98
Aqueous, 1 mg./ml., pH 5–6, unbuffered	3 mo. at 45°	74	28	102

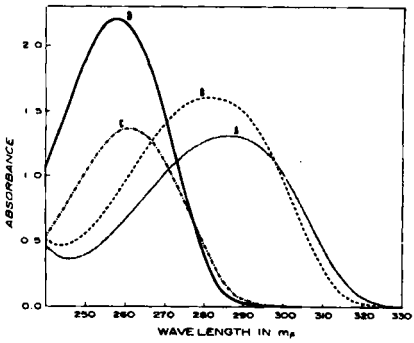

Fig. 2.—The U.V. absorption spectra of A, idoxuridine (0.06 mg./ml.); B, 5-iodouracil (0.06 mg./ml.); C, 2′-deoxyuridine (0.025 mg./ml.); D, uracil (0.025 mg./ml.) in 0.1 *N* hydrochloric acid, 1-cm. cells.

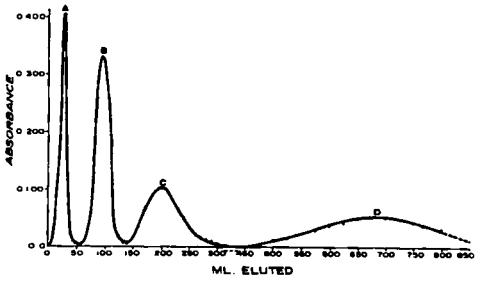

Fig. 3.—Partition column chromatogram of a mixture approximately 0.003 *M* in A, 5-iodouracil; B, idoxuridine; C, uracil; D, 2′-deoxyuridine. Column diameter = 26 mm.

only aqueous standing phases were considered. Idoxuridine is not ionized appreciably in acid solution and therefore is more readily extracted by organic solvents. This fact and its decreased stability in basic solutions led to the choice of 0.1 *N* HCl as the standing phase.

For use as the moving phase, carbon tetrachloride, chloroform, *n*-butylacetate, isooctane, and *n*-butanol were tried. None of these served well alone. Of the mixtures tried, the best separation for the lowest elution volume was given by a solvent consisting of 5 vol. of chloroform and 1 vol. of *n*-butanol.

For convenience and ease of handling, a 30-cm.-long column packed with a bed of Celite about 10 cm. long was chosen. One such column which gave a complete separation from all decomposition had a diameter of 26 mm. and a chromatographic packing of 8 ml. of 0.1 *N* HCl adsorbed on 8 Gm. of Celite 545. A typical chromatogram of an approximately equimolar synthetic mixture is shown in Fig. 3. It will be seen that these constituents are well separated and that determination of each one is accomplished readily by simple U.V. absorption spectrophotometric measurements.

The above method suffers from requiring a high elution volume (and time) and a relatively large amount of Celite. A satisfactory compromise between these factors and the degree of separation was obtained by employing a smaller column (19-mm. diameter) packed with 4 ml. of 0.1 *N* HCl adsorbed on 4 Gm. of Celite 545. Figure 4 shows typical chromatograms obtained with this setup for a synthetic mixture and for a partly decomposed sample. It is apparent from Curve A that the separation from uracil may not always be complete. However, idoxuridine can still be determined by U.V. absorption, provided a properly selected two-point calculation method is used. The U.V. absorption spectra of the eluted species are shown in Fig. 5. The method reported in detail below is the one that was used in many assays.

PROPOSED METHOD

Reagents.—Hydrochloric acid, 0.1 *N*; chloroform, Merck reagent; *n*-butanol, Merck reagent; and Celite 545, acid-washed, Johns-Manville Co., were used. Eluting solvent: mix 5 vol. of chloroform with 1 vol. of *n*-butanol.

Apparatus.—A Cary model 11 M recording spectrophotometer was used. The chromatographic column was approximately 19 mm. in diameter × 30 cm. high with a stopcock for controlling the flow rate.

Idoxuridine Standard.—Idoxuridine (California Corp. for Biochemical Research) was recrystallized twice from an aqueous methanol system. This material was shown to be 99.3% pure by nonaqueous titration, 99.6% by iodine determination, and 99.6% by phase solubility analysis. In addition, its

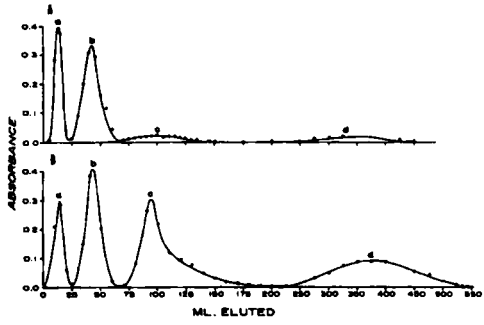

Fig. 4.—Partition column chromatograms with column diameter = 19 mm. Curve A, a heat-decomposed solution of idoxuridine; curve B, a synthetic mixture approximately 0.003 *M*. Key: *a*, 5-iodouracil; *b*, idoxuridine; *c*, uracil; *d*, 2′-deoxyuridine.

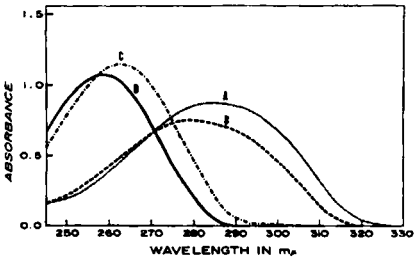

Fig. 5.—The U.V. absorption spectra of A, idoxuridine; B, 5-iodouracil; C, 2′-deoxyuridine; D, uracil, all 0.6 × 10^{-4} *M* in chloroform–*n*-butanol (5:1 by volume), 2-cm. cells.

TABLE IV.—CHROMATOGRAPHIC RESULTS FOR A 0.1% AQUEOUS SOLUTION OF IDOXURIDINE

Analyst	Assay, % of Theory
A	100.4, 97.6
B	98.2, 100.1
C	99.4, 99.8
D	100.5, 99.7

specific rotation $[\alpha]_D^{26}$, was +28.6° (C = 1, *N* NaOH). Its U.V. absorption spectrum exhibited a maximum at 288 mμ (ϵ = 7763) and a minimum at 247 mμ (ϵ = 2093).

Assay Procedure.—Add 4 ml. of 0.1 *N* HCl to 4 Gm. of Celite in a 4-oz. mortar and mix well using a small plastic rod or spatula. The mixture has been worked sufficiently when it appears light, fluffy, and uniform. Transfer this mixture to a chromatographic tube which contains a small pledget of glass wool, and tamp down with a rod of slightly smaller diameter using only sufficient force to give the packing a uniform appearance. Add a 2.00-ml. aliquot of an aqueous sample (pH < 7, concentration 0.5–1 mg. idoxuridine/ml.) to 2 Gm. of Celite in a 4-oz. mortar, mix as before, and transfer to the same tube; do not tamp it down yet. Add 2 ml. of 0.1 *N* HCl to 2 Gm. of Celite in the same mortar used for the sample and mix well. Transfer about half of this to the same tube and tamp as before until the packing appears to be uniform. Then transfer the remaining portion to the tube, pack it in place, and top with a small pledget of glass wool.

Pass chloroform-butanol eluting solvent through the column at a flow rate of approximately 1 ml. per minute. Since the exact nature of the elution profile may vary slightly from lot to lot of Celite, it will be necessary to run a known idoxuridine solution (0.5–1.0 mg./ml.) through the procedure to determine exactly what part of the eluate contains idoxuridine. Collect and save for spectrophotometric examination that portion of the eluate containing all of the idoxuridine. For most lots of Celite, collect a fore-run of 15–20 ml. and discard. Then place a 100-ml. volumetric flask under the column and collect the next 80 ml. Dilute to volume with the same solvent. Record the U.V. absorption spectrum *versus* this solvent in 2-cm. cells from 320 to 260 mμ.

$$\text{Idoxuridine, mg./original sample, ml.} = \frac{(\text{Å}_{.292} - \text{Å}_{.310})\text{sample}}{(\text{Å}_{.292} - \text{Å}_{.310})\text{std.}} \times \text{concn. of std. (mg./ml.)}$$

RESULTS

Some typical results obtained with the chromatographic method are shown in Table IV. The standard deviation for these values is 1.0%, although experience has shown that a more realistic value is 1.3%.

That this method is a stability method of assay is amply demonstrated by all of the chromatographic evidence and the results given in Table III. This can also be demonstrated by assaying known solutions of idoxuridine where the breakdown products have been added; *e.g.*, a mixture of 95.3 mg. idoxuridine, 14.8 mg. uracil, and 13.5 mg. iodouracil was assayed, and the average result was 95.2 mg. idoxuridine (99.9% recovery). Similarly, deoxyuridine was shown to cause no interference.

DISCUSSION

The method presented above appears to have no especially critical steps. No care is taken to store Celite protected from the atmosphere; acid-washing will remove a small background absorbance, but this is usually unnecessary. The absorptivity for idoxuridine dissolved directly in chloroform-butanol eluting solvent is identical with that obtained by putting a known aqueous solution through the chromatographic procedure; this indicates that there is no retention or destruction on the column. However, if the mixing of Celite with standing phase is accomplished with vigorous grinding, a material of pastelike consistency is obtained. This results in considerably lower recoveries.

With minor modification, the chromatographic method has been applied to other pharmaceutical forms and mixtures containing other drug substances. For example, in the presence of hydrocortisone, prednisolone, or dimethisoquin, which would normally interfere, the loaded column is given a prewash of chloroform prior to normal chromatographic development. Some materials such as neomycin sulfate are so soluble in the standing phase that they normally do not interfere.

All of the data indicate that this procedure is sufficiently accurate and precise for assaying freshly prepared, aged, or decomposed solutions, or mixtures of idoxuridine in a wide variety of situations.

SUMMARY

Ultraviolet light reacts with aqueous solutions of idoxuridine in a way which destroys its characteristic U.V. absorption spectrum. This suggests that the pyrimidine ring has been hydrated or opened.

Light from a sunlamp reacts with aqueous idoxuridine solutions to produce two major products: 2′-deoxyuridine and material whose pyrimidine ring has been altered.

The thermally induced hydrolysis products of idoxuridine are principally 5-iodouracil and 2-deoxyribose, with a trace to 2′-deoxyuridine. In time these will lead to the formation of uracil.

A column partition chromatographic procedure has been developed for the assay of idoxuridine in the presence of its decomposition products.

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