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THE DEVELOPMENT OF BRITISH PHARMACOPOEIA MONOGRAPHS FOR IDOXURIDINE AND IDOXURIDINE EYE DROPS USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY FOR ASSAY AND FOR CONTROLLING RELATED IMPURITIES

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SUMMARY

The monograph published in the 1973 edition of the British Pharmacopoeia (BP) for idoxuridine required revision because it contained a non-specific assay and no tests for related impurities. It was also necessary to prepare a new monograph for idoxuridine eye drops. The Japanese Pharmacopoeia contains a thin-layer chromatographic test for impurities but this was not considered ideal. Improved thin-layer chromatographic tests were sought but when this was unsuccessful, methods using high-pressure liquid chromatography were examined. A system using reversed-phase chromatography was selected for inclusion in BP Addendum 1977 since it provided a specific assay method and limit test for related impurities which could be applied to both the drug substance and the eye drops.

INTRODUCTION

Idoxuridine (5-iodo-2′-deoxyuridine) has specific anti-viral properties inhibiting the formation of DNA in virus infected cells and thus preventing replication in species such as Adenovirus, Cytomegalovirus, Herpes simplex and Vaccinia[1]. Accordingly it is used in the form of eye drops and eye ointment for the treatment of virus-induced conditions such as herpetic keratitis.

A monograph for idoxuridine was first published in the British Pharmacopoeia (BP) 1973 edition[2], but it was necessary to improve this because the assay used was non-specific being based on the determination of iodine and no control of possible related impurities was applied. In addition it became necessary to develop a monograph for idoxuridine eye drops.

Monographs for idoxuridine are also included in the United States Pharmacopeia (USP)[3] and Japanese Pharmacopoeia[4]. The assay in the USP monograph like that in BP1973 is non-specific, in this case being a non-aqueous titration with sodium methoxide and again no specific test for related impurities is included. The assay in the Japanese Pharmacopoeia monograph is also a sodium methoxide titration but in

this case a test to control impurities by thin-layer chromatography (TLC) is included. This test involves two-dimensional chromatography of a 50-μg sample of idoxuridine on a fluorescent chromatoplate and the requirement is that no secondary spots shall be visible when inspected under ultraviolet radiation at 254 nm. This test was considered unsatisfactory for the following reasons:

(i) It is undesirable to stipulate that no secondary spots should be visible since the result becomes dependant on such factors as the eyesight of the analyst or the nature of the equipment used in which the assessment is carried out. In BP monographs a standard is usually applied to the chromatoplate. This may be a sample of the impurity in question at the maximum permitted level or a dilution of the material under examination with the requirement that the intensity of any secondary spots in the sample are no greater than the standard.

(ii) It is considered undesirable to use two-dimensional chromatography for a test of this nature. This mode of separation inevitably results in greater diffusion of spots leading to loss of sensitivity. Two-dimensional separations also suffer from the disadvantage that standards cannot be satisfactorily applied to the same plate as the sample under test.

(iii) TLC work carried out at the BP laboratory indicated that for one of the likely impurities, 5-iodouracil, the minimum level that could be assessed using 254-nm radiation after a one-dimensional separation was 1 μg, which would correspond to a 2% level in the Japanese Pharmacopoeia test. In the BP monographs it was desired to control impurities at 0.5% levels.

The degradation pathways of idoxuridine have been studied by Ravin *et al.*[5] and Simpson and Zappala[6], and the main routes are illustrated in Fig. 1. They found that the principal effect of light-induced degradation was loss of iodine leading to

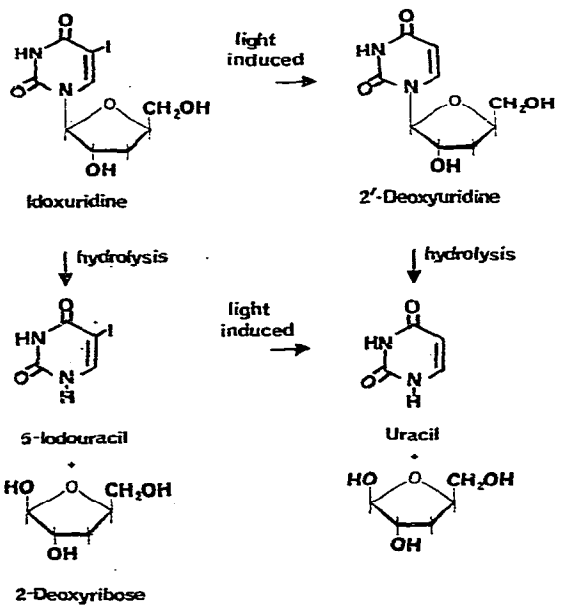


Fig. 1. Degradation pathways of idoxuridine.

2'-deoxyuridine although intense ultraviolet irradiation gave rise to some unknown product which they thought was probably due to breakdown of the pyrimidine ring. In aqueous solution, the principal products of heat-induced degradation were 5-iodouracil and 2-deoxyribose, only low levels of 2'-deoxyuridine being reported by this route. This result was found at any pH, but the rate of degradation was far more rapid under basic conditions. The generation of uracil by either route was extremely slow. Degradation studies were monitored by paper chromatography and under the conditions used, uracil was only detected in samples that had decomposed to an extent of about 50%.

These workers devised a method of separating idoxuridine from its impurities by partition chromatography on a Celite column impregnated with 0.1 *M* hydrochloric acid and monitoring the eluate spectrophotometrically at 292 nm. This method forms the basis of the assays for the Idoxuridine Ophthalmic Solution and Eye Ointment monographs in the USP[3]. This was not considered satisfactory in the present study for the following reasons:

(i) The method was not considered sufficiently robust for a pharmacopoeial test since variations in the nature of columns prepared in different laboratories and between different batches of Celite would be expected to create difficulties in obtaining repeatability between laboratories. These difficulties may well be overcome by those with experience of this procedure but pharmacopoeial tests are often required for use on a "one off" basis and are designed accordingly.

(ii) Although this represents an improvement on the monograph in BP1973 since the assay is specific for idoxuridine, it is doubtful whether the precision of such a procedure would allow an analyst to discriminate against samples containing contaminants above the 0.5% level.

What was needed was a specific test for these impurities such as a TLC limit test. If this were unsuccessful high-pressure liquid chromatography (HPLC) would be investigated with a view to providing both specific assays for the drug substance and the eye drops and also specific tests for impurities in the two monographs.

EXPERIMENTAL

Thin-layer chromatography

TLC was carried out on 20 × 20 cm glass-backed chromatoplates either hand prepared with a 250-μm layer of silica gel Type 60 GF or HF (Merck, Darmstadt, G.F.R.) or were precoated silica gel 60 F_{254} (Merck). Spots were located by viewing under shortwave ultraviolet radiation (254 nm).

High-pressure liquid chromatography

A Waters Assoc. (Milford, Mass., U.S.A.) ALC 202 chromatograph fitted with an M-6000A solvent delivery system, a U 6K injector and a 440 ultraviolet detector operating at 254 nm were used. Normal phase chromatography was carried out on a 30 cm × 4 mm I.D. column packed with 10-μm silica particles (μPorasil, Waters Assoc.). Reversed-phase work was carried out on a 30 cm × 4 mm I.D. column packed with 10-μm silica particles modified with chemically bonded octadecasilyl groups (μBondapak C_{18}, Waters Assoc.).

Materials

Samples of idoxuridine and Kerecid eye drops containing 0.1% (w/v) idoxuridine and 0.002% (w/v) sodium thiomersalate preservative were kindly provided by Smith Kline & French (Welwyn Garden City, Great Britain). Samples of 5-iodouracil, 2'-deoxyuridine and uracil were obtained from Sigma (St. Louis, Mo., U.S.A.). All organic solvents were AnalaR grade obtained from BDH (Poole, Great Britain) and were used without any further treatment. Mobile phases for reversed-phase work were prepared using glass distilled water and were de-aerated by stirring for about 15 min under reduced pressure.

RESULTS AND DISCUSSION

Thin-layer chromatography

Several different systems were investigated of which one showing promise used hand-prepared chromatoplates and chloroform–methanol–acetic acid (8:1:1) as the mobile phase. TLC results are tabulated in Table I and illustrated in Fig. 2.

TABLE I

R_F VALUES OF IDOXURIDINE AND IMPURITIES IN SOME TLC SYSTEMS

Mobile phase	*5-Iodouracil*	*Uracil*	*Idoxuridine*	*2'-Deoxyuridine*
Chloroform–methanol–acetic acid (8:1:1)	0.64	0.39	0.30	0.16
Isopropanol–conc. ammonia (9:1), unsaturated	0.42	0.35	0.15	0.20
Isopropanol–conc. ammonia (9:1), saturated	0.30	0.27	0.09	0.16

The minimum loading that could be assessed by the quenching of 254-nm radiation was about 1 μg for 5-iodouracil and 0.5 μg for 2'-deoxyuridine so a loading of 200 μg idoxuridine would be required to control impurities at the 0.5% level. Compact spots for idoxuridine could only be achieved in this system when loadings no greater than 25 μg were applied to the chromatoplate. Tailing spots were obtained with higher levels and since the 2'-deoxyuridine spot ran behind the principal spot, the tailing interfered with the assessment of this impurity.

This difficulty was partially overcome by an alternative system using precoated chromatoplates and 2-propanol–concentrated ammonia (9:1) as the mobile phase under unsaturated tank conditions[7]. As can be seen from Table I and Fig. 2 the change from the acidic to the basic system did not affect the order of retention of 5-iodouracil and uracil, but that of idoxuridine and 2'-deoxyuridine was reversed so that the impurity was no longer in the tail of the principal component. It was also found that this system gave rise to far less tailing than was experienced with the acidic system and 200 μg of idoxuridine could readily be chromatographed.

This system suffered from two disadvantages:

(1) The requirement that an unsaturated system be used. For official methods that are designed to be used in a number of different laboratories it was considered that greater reproducibility of results is likely to be achieved under saturated conditions. With unsaturated systems the composition of the atmosphere within the tank

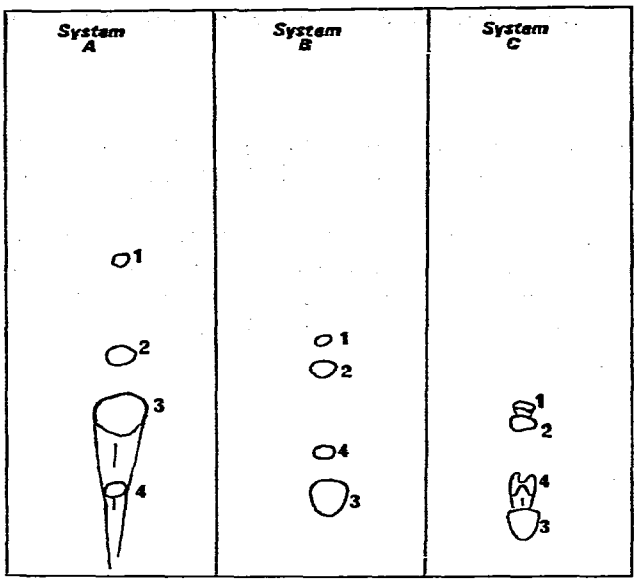


Fig. 2. Thin-layer chromatograms of idoxuridine and its impurities. System A: chromatoplate, silica gel 60 HF; mobile phase, chloroform–methanol–acetic acid (8:1:1). Sample: 10 μl of a solution in methanol containing 0.01% each of 5-iodouracil (spot 1), uracil (spot 2) and 2′-deoxyuridine (spot 4) and 1.0% of idoxuridine (spot 3). System B: chromatoplate, silica gel 60 F254 precoated plate; mobile phase, isopropanol–conc. ammonia (9:1) under unsaturated tank conditions. Sample: 4 μl of a solution in methanol–conc. ammonia (5:1) containing 0.025% each of 5-iodouracil (spot 1), uracil (spot 2), 2′-deoxyuridine (spot 4) and 5% of idoxuridine (spot 3). System C: same as System B but under saturated tank conditions.

is likely to be subject to greater variation from laboratory to laboratory which could result in variations in the degree of separation achieved.

This method was attempted under saturated conditions and as is evident from Table I and Fig. 2 the retention of components were reduced and the separation was poorer. Apart from this, the 2′-deoxyuridine no longer ran as a compact spot but instead an "H" shaped spot which tailed into the principal spot resulted.

It would appear from this that under saturated conditions the activity of the silica in this separation was increased. A possible explanation is that under unsaturated conditions separations were obtained that were better than those achieved in acidic systems because of the greater solubility of idoxuridine in aqueous or alcoholic bases. Under saturated conditions, however, the silica surface was modified by the ammonia in the system and it too was rendered basic leading to a greater affinity of the components for the standing phase.

(2) The minimum visible level of 5-iodouracil was about 1 μg and so to control this impurity at the 0.5% level it was necessary to chromatograph 200 μg of idoxuridine. For the drug substance this presented no difficulty since a 5% solution could be prepared in methanol–concentrated ammonia (5:1) requiring 4-μl applications to the chromatoplate. The eye drops being a 0.1% solution would require either a 200-μl application or a preliminary concentration of the solution, a process which in itself could lead to some degradation.

For these reasons it was considered that there was adequate justification in applying HPLC to this analysis.

High-pressure liquid chromatography

Following on from the work done on TLC, preliminary HPLC work was carried out on a silica column using a mobile phase composed of chlorinated hydrocarbon and methanol. Chloroform was not used because of the ethanol stabiliser which often varies from batch to batch. Dichloromethane was found to be a suitable alternative. Good separations were obtained on the μPorasil column with dichloromethane–methanol (95:5) as the mobile phase at a flow-rate of 2.0 ml per minute. Under these conditions, capacity ratios (k' values) as shown in Table II were obtained and a typical chromatogram is shown in Fig. 3.

TABLE II

k' VALUES FOR IDOXURIDINE AND ITS DEGRADATION PRODUCTS USING SILICA COLUMN

Component	*k' value*
5-Iodouracil	0.57
Idoxuridine	2.14
Uracil	3.76
Unknown	4.38
2'-Deoxyuridine	6.14

Although this system seemed promising for testing the drug material, it was not satisfactory for the eye drops. Since this was an aqueous solution, it could not be injected directly onto the column. The alternative was a preliminary treatment such as evaporation to dryness and dissolution of the residue in a non-aqueous solvent. This too was undesirable since it added to the analysis time and these was some risk of degradation during the evaporation stage. What was required to avoid this difficulty was a system using a chemically modified silica such as one using reversed-phase chromatography.

This was satisfied using the μBondapak C_{18} column on which an aqueous methanolic mobile phase was suggested to us[8]. Good results were obtained using water–methanol (87:13) at a flow-rate of 1.7 ml/min and under these conditions, the k' values given in Table III resulted and a typical chromatogram is shown in Fig. 4.

It is of interest to note that the use of a reversed-phase system did not result in a reversal of the order of elution of all peaks. The components of this system could be considered in two different groups. Uracil and 5-iodouracil are pyrimidines, idoxuridine and 2'-deoxyuridine are nucleosides. It is probable that the various factors leading to separation contribute to different extents within these two groups. As a result the order of elution of uracil and 5-iodouracil was reversed on the two columns as also was the order of elution for idoxuridine and 2'-deoxyuridine. This effect led to a further advantage of the reversed-phase system. It was hoped to use this system for controlling the level of impurities in idoxuridine and for this purpose it was proposed to use sensitivities that would lead to off-scale idoxuridine peaks. In the silica system,

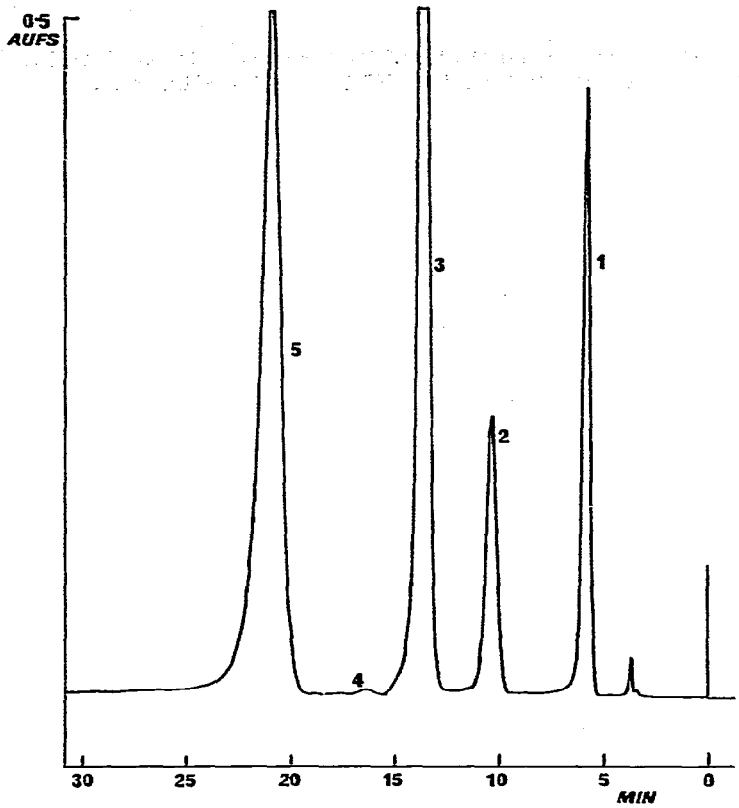


Fig. 3. Liquid chromatogram of idoxuridine and its impurities. Column: 30 cm × 4 mm I.D. μPorasil. Eluent dichloromethane–methanol (95:5). Flow-rate: 2 ml/min. Sample: 4 μl of a solution in methanol containing 0.7% each of 5-iodouracil (peak 1), idoxuridine (peak 2), uracil (peak 3), 2′-deoxyuridine (peak 5). Peak 4 is an unknown impurity.

the 2′-deoxyuridine would have eluted in the tail of this principal peak which would have adversely affected the accuracy of the test. In the reversed-phase system, all the impurity peaks eluted before the principal peak.

Assay

The system was first examined for its suitability as an assay method both for the drug substance and the eye drops. For this assay it had been suggested to us that sulphathiazole be used as the internal standard[8]. In this system sulphathiazole eluted just after idoxuridine as indicated in Table III. The sample of eye drops available for examination contained thiomersalate preservative, but it is believed that benzalkonium chloride may also be used. To test the method it was necessary to examine the linearity, the precision and whether preservatives would interfere.

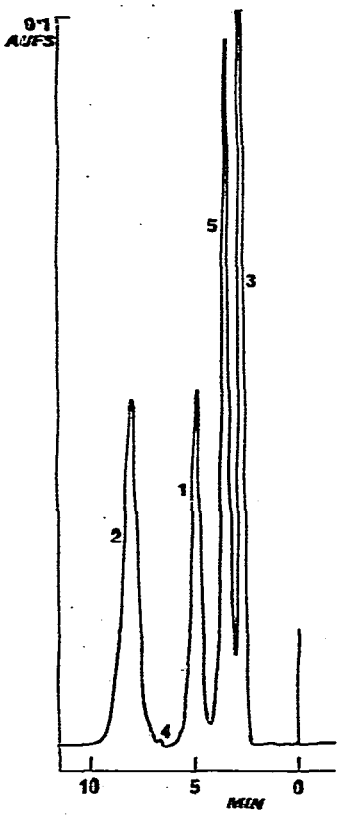
Linearity

For eye drops, pharmacopoeial monographs typically allow assay limits of 90 to 110% of the declared content. To include the region of interest, the linearity

TABLE III

k' VALUES FOR IDOXURIDINE AND ITS DEGRADATION PRODUCTS USING REVERSED-PHASE SYSTEM WITH WATER–METHANOL (87:13) AS MOBILE PHASE

Component	*k' value*
Uracil	1.90
2'-deoxyuridine	2.76
5-Iodouracil	4.52
Unknown	5.80
Idoxuridine	7.90
Sulphathiazole	10.60



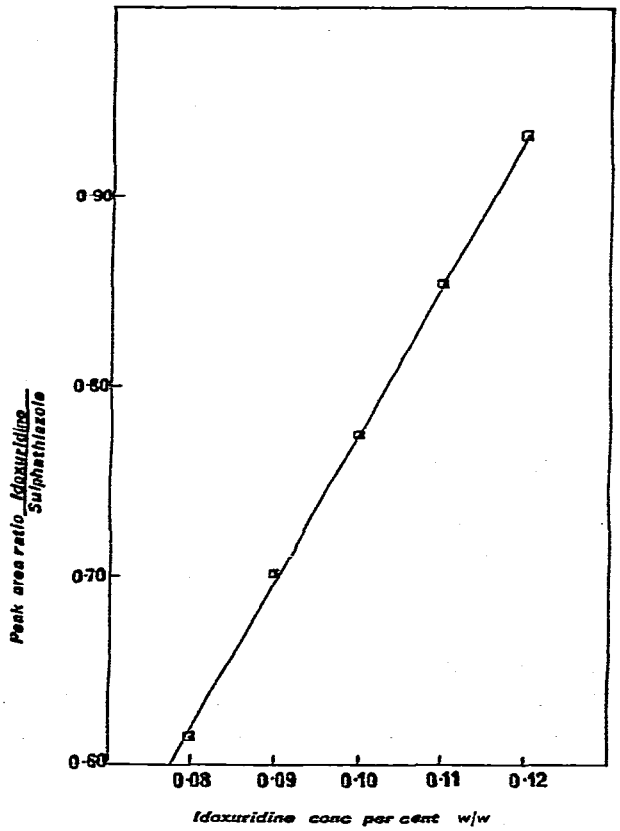


Fig. 4. Liquid chromatogram of idoxuridine and its impurities. Column: 30 cm × 4 mm I.D. μBondapak C_{18}. Eluent: water–methanol (87:13). Flow-rate: 1.7 ml/min. Sample: 10 μl of a solution in water containing 0.034% of 5-iodouracil (peak 1), 0.080% of idoxuridine (peak 2), 0.006% uracil (peak 3), 0.013% 2'-deoxyuridine (peak 5). Peak 4 is an unknown impurity.

Fig. 5. Plot of ratio idoxuridine peak area/sulphathiazole peak area *versus* idoxuridine concentration to demonstrate linearity of assay.

was tested from 80 to 120%. Solutions were prepared containing 0.08, 0.09, 0.10, 0.11 and 0.12% idoxuridine in water. To 15-ml aliquots, were added 2 ml of a 0.12% solution of sulphathiazole in 10% aqueous ethanol the volumes were then made up to 20 ml with water. Aliquots (15 μl) of each solution were injected in duplicate and

TABLE IV

PEAK AREA RATIOS IDOXURIDINE/SULPHATHIAZOLE FOR A SERIES OF IDOXURIDINE CONCENTRATIONS

Concentration of idoxuridine (%)	*Ratio of peak areas idoxuridine/sulphathiazole*	*Mean*
0.08	0.6163	0.6146
	0.6128	
0.09	0.7064	0.7010
	0.6956	
0.10	0.7736	0.7740
	0.7745	
0.11	0.8528	0.8537
	0.8546	
0.12	0.9305	0.9310
	0.9315	

the areas of the peaks due to idoxuridine and sulphathiazole determined. The results are shown in Table IV and the linearity is demonstrated in Fig. 5.

Precision

To test the precision of the assay a 0.10% solution of idoxuridine was prepared in water. To a 15-ml aliquot was added 2 ml of a 0.12% solution of sulphathiazole in 10% aqueous ethanol and the volume was made up to 20 ml with water. Six replicate aliquots of 15 μl were analysed and the areas of the peaks due to idoxuridine and sulphathiazole determined. The mean and coefficient of variance were determined as shown in Table V.

TABLE V

DETERMINATION OF PRECISION OF ASSAY USING ALIQUOTS OF A 0.1% SOLUTION OF IDOXURIDINE

Sample	*Ratio of peak areas idoxuridine/sulphathiazole*
1	0.7262
2	0.7249
3	0.7307
4	0.7232
5	0.7197
6	0.7201
Mean	0.7242
Standard deviation	0.0041
Coefficient of variance	0.57

Interference by preservatives

The sample of eye drops available for examination contained 0.002% thiomersalate as a preservative. It was found that if an aqueous solution of sodium thiomersalate was injected into the chromatograph under the conditions of the assay, it eluted just after the inert peak and would therefore cause no interference. When formulated into the eye drops, over a period of time, thiomersalate degrades giving

rise to a series of slightly more retained peaks; however, these still give rise to no interference and Fig. 6 shows a typical separation of a sample of eye drops.

If benzalkonium chloride is used as a preservative, under the assay conditions it is retained on the column and the levels normally present when used as a preservative would not be expected to lead to any interference; however, to be sure of this, after a few analyses, any benzalkonium present may be washed off the column with methanol.

Assay method

The following procedure is used to assay idoxuridine or idoxuridine eye drops. Prepare a 0.1% solution of the sample in water. To a 15-ml aliquot of the sample add 2 ml of a solution of sulphathiazole prepared by dissolving 120 mg of the sulphonamide in 10 ml of ethanol and diluting to 100 ml with water. Dilute this solution to 20 ml with water. Aliquots of this solution and a similarly prepared standard are injected into the chromatograph and results may be determined by measuring peak areas or peak heights. The time taken for an analysis is about sixteen minutes.

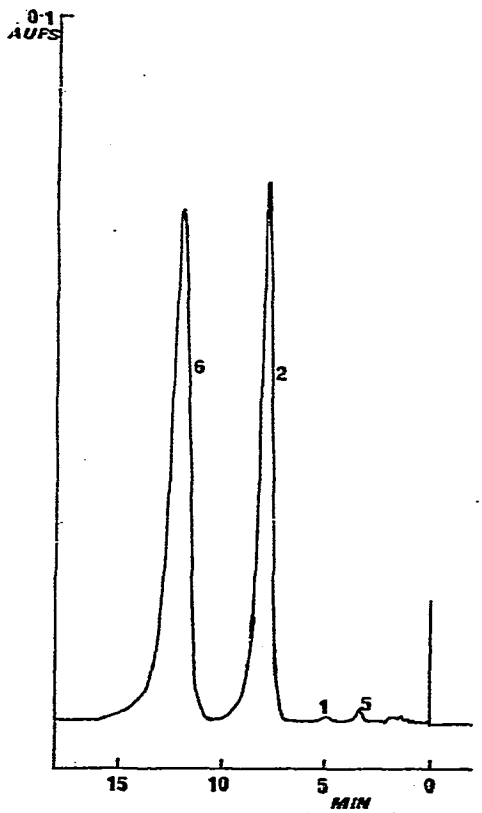


Fig. 6. Liquid chromatogram of idoxuridine eye drops. Conditions as in Fig. 4. Sample: 10 μl of a solution in water containing 0.075% idoxuridine (peak 2) and 0.012% sulphathiazole (peak 6). Small peaks due to 5-iodouracil (peak 1) and 2′-deoxyuridine (peak 5) are also visible.

Determination of impurities

The assay described above may be regarded as specific for idoxuridine since related impurities do not contribute to the figures obtained in the determination. It is currently popular to refer to an assay of this type as being "stability indicating". It is apparently considered that degradation of the material of interest will lead to a reduction of its peak size and this reduction may then be used to monitor the formation of degradation products and therefore the stability of the material under investigation. This approach was considered unsatisfactory for our purposes because:

(1) We proposed to limit impurities at the 0.5% level. It was shown earlier that the coefficient of variance for the assay was 0.57. The assay therefore did not have sufficient precision to detect 0.5% levels of degradation with a sufficiently high confidence level.

(2) Pharmacopoeial assay standards for formulations of the type under consideration customarily allow ranges of 90.0 to 110.0% of the stated amount. The intention here is to allow for batch to batch variations that are likely during production and not for the subsequent degradation of the material. If this were the case, a batch could be prepared assaying at 0.11% idoxuridine and after a prolonged period of storage the assay figure could fall to 0.09%. On both occasions the material would satisfy the requirement in spite of about 18% degradation having occurred. This is, of course, unacceptable and for these reasons tests that specifically measure the levels of degradation products are preferred.

In this case, the impurities of interest were 5-iodouracil, 2'-deoxyuridine and uracil. As was mentioned earlier, it had been demonstrated that compared with the former materials, uracil is generated only very slowly being a secondary product of degradation[6]. It was felt that before any significant amounts of uracil were formed, a sample would contain well over the allowed level of the more readily formed 5-iodouracil or 2'-deoxyuridine. For this reason it was considered adequate to limit the levels of these two contaminants.

As is apparent from Table III and the chromatogram shown in Fig. 4, the chromatographic conditions used in the assay provided an adequate separation of these impurities. The difficulty here was the internal standard. The intention was to inject larger quantities of idoxuridine and to use higher sensitivities in order to produce appreciable responses from low levels of the impurities of interest. The internal standard would be expected to produce a comparable response but the idoxuridine peak would be well off scale. It was undesirable for the internal standard

TABLE VI

k' VALUES FOR IDOXURIDINE AND ITS DEGRADATION PRODUCTS USING REVERSED-PHASE SYSTEM WITH WATER–METHANOL (96:4) AS MOBILE PHASE

Component	*k' value*
Uracil	2.64
Sulphanilamide	4.25
2'-Deoxyuridine	5.66
5-Iodouracil	8.36
Unknown	14.23
Idoxuridine	20.82

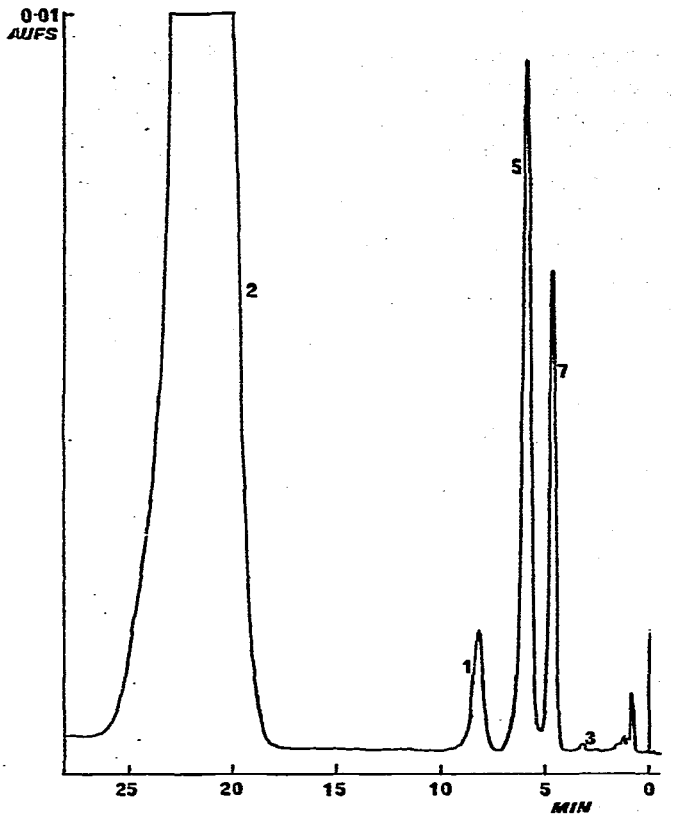


Fig. 7. Liquid chromatogram of idoxuridine. Column: 30 cm × 4 mm I.D. μBondapak C_{18}. Eluent: water–methanol (96:4). Flow-rate: 1.7 ml/min. Sample: 15 μl of a solution in water containing 0.08% idoxuridine (peak 2), 0.0004% each of 5-iodouracil (peak 1) and 2′-deoxyuridine (peak 5), 0.0001% of sulphanilamide (peak 7). A trace of uracil is also visible (peak 3).

to elute in the tail of the principal peak but ideally it would elute close to the peaks of interest in this test. For this reason sulphathiazole was considered unsatisfactory and of the alternative materials examined, sulphanilamide gave the most promising results, eluting just before the 2′-deoxyuridine. For optimum resolution the solvent was weakened to water–methanol (96:4) and under these conditions the k' values for idoxuridine and its impurities were as shown in Table VI.

Figs. 7 and 8 show chromatograms of idoxuridine and idoxuridine eye drops respectively containing 0.5 and 0.4% levels of 5-iodouracil and 2′-deoxyuridine. This method was tested by preparing a 0.1% solution of idoxuridine. To 16 ml were added 2 ml of a 0.001% aqueous solution of sulphanilamide and the solution was diluted to 20 ml. Similar solutions were prepared but also made to contain 0.0002, 0.0004 and 0.0008% of 5-iodouracil and 2′-deoxyuridine which was equivalent to 0.25, 0.50 and 1.00% levels of these impurities in the idoxuridine. Aliquots (25 μl) of these solutions were chromatographed in duplicate and from the areas of the peaks due to the internal standard and impurities the recovery of impurities was determined. The results are given in Table VII. For the purposes of a limit test for these impurities these results were considered to be quite satisfactory.

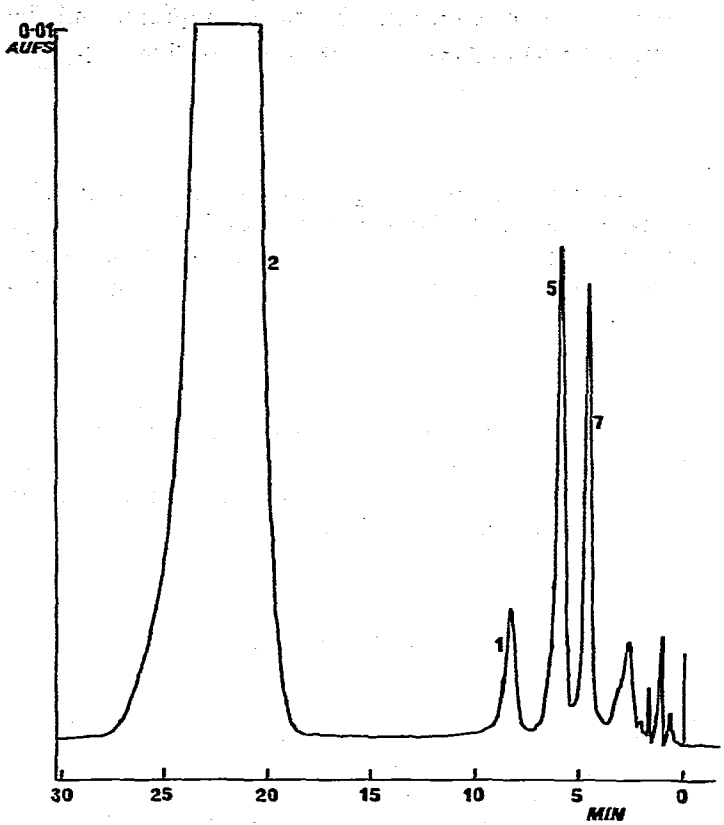


Fig. 8. Liquid chromatogram of idoxuridine eye drops. Conditions as in Fig. 7. Sample as in Fig. 7 except concentrations of 5-iodouracil and 2-deoxyuridine are 0.0003%. Some peaks due to thiomersalate and its degradation products appear before the sulphanilamide peak. Peak identities are as in Fig. 7.

TABLE VII

DETERMINATION OF ACCURACY–RECOVERY OF 5-IODOURACIL AND 2'-DEOXYURIDINE FROM SAMPLES OF IDOXURIDINE SPIKED WITH THE IMPURITIES

Level of impurity added (%)	*5-Iodouracil*		*2'-Deoxyuridine*	
	Found (%)	*Recovery (%)*	*Found (%)*	*Recovery (%)*
0	0.06	—	0.08	—
0.25	0.28	88.0	0.32	96.0
0.50	0.56	100.0	0.60	104.0
1.00	1.04	98.0	1.10	102.0

Method for impurity test

The procedure for the impurity test is as follows. Prepare a 0.1% solution of the sample in water. To 16 ml add 2 ml of a 0.001% aqueous solution of sulphanilamide and dilute to 20 ml with water. A standard solution is prepared containing 0.0004% of 5-iodouracil and 2'-deoxyuridine and 0.0001% of sulphanilamide.

Aliquots of the sample and standard are injected into the chromatograph and the levels of impurities determined by measuring peak areas or peak heights. The time taken for an analysis is about 25 min.

CONCLUSION

An HPLC system using reversed-phase chromatography has been successfully developed and incorporated into BP monographs for idoxuridine and idoxuridine eye drops[9]. This method provides the monographs both with specific assay methods and with tests for controlling the levels of impurities that may arise either during manufacture or as a result of degradation.

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