DETERMINATION OF ASSAY AND IMPURITIES OF GAMMA IRRADIATED CHLORAMPHENICOL IN EYE OINTMENT

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Abstract

A sample preparation method was developed to isolate chloramphenicol and its radiolytic products from an oily ointment base. The isolation method suspended the eye ointment in n-hexane at 45 °C, and isolated the target compounds as residue by centrifugation. It was found that the main element to ensure a satisfactory isolation was keeping the sample solution at 45°C during sample preparation. Linearity, precision, accuracy and suitability of the method were confirmed valid for both assay and impurity tests. This isolation method was ideal for assay, unique for extraction of unexpected and complex radiolysis products, and had a number of advantages compared to the pretreatment methods described in the United States Pharmacopoeia and British Pharmacopoeia, in terms of accuracy, precision, and easy handling. The effect of γ-irradiation on chloramphenicol eye ointment was studied by HPLC-DAD, after applying the developed sample preparation method. The present assay and impurity test methods with HPLC-DAD were confirmed to be suitable for irradiated chloramphenicol in eye ointment.

1. INTRODUCTION

Chloramphenicol (CAP) was initially determined by microbiological assay, but the elucidation of its structure has led to the use of a wide variety of chemical and physicochemical assay methods including argentometric titration, colourimetry, thin layer chromatography, UV spectroscopy, polarography, gas chromatography and the high-performance liquid chromatographic (HPLC). HPLC for the assay of CAP is superior to other conventional methods in speed, precision, specific and ease of performance.

Reliable determination of the influence of γ-irradiation on chloramphenicol in eye ointment depends critically on proper isolation of CAP and its possible degradation products from the ointment base. Liquid-liquid extraction, solid-phase extraction and centrifugation are generally applied for separating chloramphenicol from matrixes. Being typical traditional isolation methods relative to on chloramphenicol eye ointment (CAPEO), the methods of The United States Pharmacopoeia (USP) and British Pharmacopoeia (BP) employ liquid-liquid extraction using methanol and water as extraction agents. Attia et al. presented an extraction method to deal with the effect of ointment bases and temperatures on the stability of chlortetracycline hydrochloride and chloramphenicol in eye ointments. Kim et al. used graphitized carbon black as solid-phase to extract CAP from biological samples.

However, liquid-liquid extraction and solid-phase extraction are generally designed for assay of general chloramphenicol products only, and may not be applied directly to investigation of radiolysis products because it could not ensure an exhausted extraction of the complex and trace radiolysis products. Separating CAP impurities from petrolatum ointment has been little studied in past.

Centrifugation, dissolving eye ointment in hydrophobic solvent and then separating the ointment part by centrifugation, can keep all the hydrophilic parts remained and ensure exhausted extraction. Although centrifugation is generally used as non-quantitative separation method, the method was used for qualitative separating neomycin from petrolatum based ointment for assay test. The aim of present work was, therefore, to explore the possibility to isolate and CAP and its radiolysis products from CAPEO and develop rapid and reliable method to determine the chemical changes of chloramphenicol eye ointment after γ-irradiation. In addition, suitability of traditional analysis methods on irradiated chloramphenicol products has yet to be confirmed.

2. EXPERIMENTAL

2.1. Material and Reagents

Chloramphenicol eye ointment, chloramphenicol powder, and eye ointment base (EOB, containing no active ingredient) were offered by Ciba Vision AG (Switzerland). All chemicals used in the present study were of reagent-grade or better. Methanol and acetonitrile were of HPLC grade solvent. The samples were irradiated in aluminium collapsible tubes by Cobalt-60 source to 25 or 50 kGy, respectively, in a radiation sterilization plant of Studer AG (Switzerland). Details of the samples in this study were summarized in Table I.

TABLE I. SAMPLE DESCRIPTION AND THEIR ABBREVIATION

	Eye Ointment Base	Chloramphenicol Powder	Chloramphenicol Eye Ointment
Non-irradiated	EOB	CAP-0	CAPEO-0
Irradiated at 25 kGy	-	CAP-25	CAPEO-25
Irradiated at 50 kGy	EOB-50	CAP-50	CAPEO-50
Spiked Samples[a]	EOB+CAP-0, EOB+CAP-50		

[a] Eye ointment base spiked with 10 mg CAP-0 and CAP-50, respectively.

2.2. Instruments and Operation Conditions

The HPLC experiments were carried out on a Merck Hitachi La Chrom liquid chromatograph equipped with an L-7100 pump, an L-7450 diode array detector, an L-7200 automatic injector, and a D-7000 interface. The operation conditions were summarized in Table II. Impurity test by HPLC was carried out according to the work of Altorfer et al. To minimize hydrolysis, all samples were analysed within 8 h after preparation.

TABLE II. HPLC EXPERIMENTAL CONDITIONS FOR ASSAY AND IMPURITY ANALYSIS

	Assay Test	Impurity Test
Column	stainless steel, 125×4mm ID	stainless steel, 250×4mm ID
Stationary Phase	LiChrospher RP 18, 5μm	LiChrospher 60 RP select B, 5μm
Mobile Phase	water:methanol:glocial acid (55:45:0.1)[a], 1.000 mL·min^{-1}	gradient: acetonitrile/phosphate buffer (20 mM, pH 2.5), 1.000 mL·min^{-1}
Detector Wavelength	280 nm	278 nm
Sampling Size	10.0 μL	20.0 μL

Gas chromatograph analysis was carried out on a Varian Star 3400 CX instrument equipped with flame ionization detector. Capillary column: Rtx-5 (crossbond® 5% diphenyl-95% dimethyl polysiloxane, BGB Analytik AG, 30m, 0.32 mm ID, 0.5μm), 50 °C (hold 1 min) to 200 °C at 5 °C·min-1.

2.3. Sample Preparation Procedures

Samples of non-irradiated/irradiated CAP powder were prepared according to the procedures described in Table III. For CAPEO samples, chloramphenicol and its degradation product were first isolated as dry powder and then prepared with the same procedures as that for CAP powder.

TABLE III. SAMPLE PREPARATION FOR THE HPLC ANALYSIS

	Assay Test	Impurity Test
Initial Amount 1	10 mg CAP or equivalent	10 mg CAP or equivalent
Dilution 1	50 mL, methanol	2 mL, mobile phase
Initial Amount 2	10 mL of Dilute 1	none
Dilution 2	50 mL, mobile phase	none

The isolation was carried out as following: equivalent to 10 mg CAP of CAPEO was accurately weighed into a 15-mL glass centrifuge tube. After adding 10-mL n-hexane, the sample was placed in water bath at 45°C for ca. 5 min and agitated until it was dissolved well. The sample was then centrifuged at 3500 rpm·min-1 for 2 min, and the supernatant liquid was discarded. This procedure was repeated three times. The analysis was carried put with the residues.

3. RESULTS AND DISCUSSION

3.1. Justification of the Method

With n-hexane as the extraction medium, the present isolation method separated successfully the eye ointment into hydrophilic and hydrophobic portions. It covered the whole hydrophilic part of CAP and its radiolysis products. CAP contained strong polar groups like intro, hydroxyl and dichloro etc., which were very active during gamma processing, therefore the radiolysis products of CAP were normally unexpected and complex. In this case, liquid-liquid extraction or solid phase extraction could not ensure the exhaustive extraction.

Leaving the n-hexane insoluble portion as dry residues, the method assured more freedom to choose solvent or solution concentration to dissolve those compounds for further analyses. This suited extremely well for the cases of analysis of radiolytic products, which were often unusual, complex and trace. This was in contrast to the methods of USP and BP, by which CAP and its degradation products would be extracted into a dilution solution of methanol or water.

The USP employed methanol as the extraction medium to separate CAP from the ointment base. It was found that white precipitates were produced in the resulting solution, which not only interfered with experimental operations of assay, but also resulted in impurity test to fail.

In addition, because CAP and its degradation products were isolated as dry powder, the present isolation made it easy to introduce other techniques (i.e. IR, TLC, NMR, LC-MS, UV etc.) for investigation of assay and radiolysis products in the ointment preparations. Finally, the manipulation of this method was very simple with only three times of centrifugation and reduced solvent consumption as well.

3.2. Linearity

Typical chromatogram of radiolytic products by the impurity test was showed in Fig. 1. Seven main impurity peaks were selected to study the impurities (identification of these peaks will be reported in our further work). Peak areas were used for quantitative calculation. In order to elicit the linearity of the present method, six levels over the range of 80-130% and 80-120% of the target concentration were used for assay test and impurity test, respectively. It was found that the peak areas were linearly related to the concentration over the given ranges in both cases. Least-squares regression analysis and statistical evaluation in Table IV showed excellent linear behavior for assay and impurity test, as all the correlation coefficients (R) are more than 0.99.

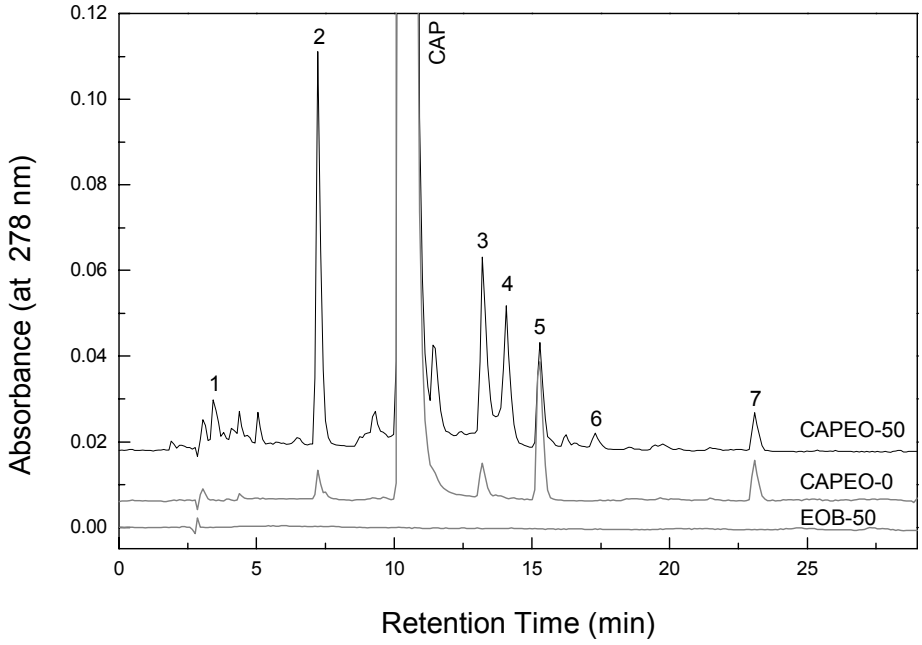

FIG. 1. Typical chromatograms of impurity test. CAPEO-0 and CAPEO-50 represent chloramphenicol eye ointment non-irradiated and irradiated at 50 kGy, EOB-50 represents eye ointment base (without active ingredient) irradiated at 50 kGy.

3.3. Precision

Precision of the isolation method was examined for assay test and impurity test, respectively. In the assay test, ointment samples including CAPEO-0, EOB+CAP-0, and CAPEO-50 were respectively isolated and analysed with six replicates. The relative standard deviation (RSD) of the final analysis results (Fig. 2), including the errors of the isolation and the HPLC procedures, fell well into the 95% confidence interval of the RSD of the HPLC determination alone (0.59 - 2.3), which were measured using chloramphenicol reference solution (excluding isolation procedure). The results indicated that experimental errors from the isolation procedure were within that from HPLC procedure, confirming the validity of sample preparation for assay test.

For impurity test, precision was determined by the sample (EOB+CAP-50) that was prepared by spiking CAP-50 into eye ointment base (EOB). Similarly, the RSD of EOB+CAP-50 included the errors of both the isolation and the HPLC procedures, while the RSD of CAP-50, going though only HPLC analysis, represented the precision of the HPLC analysis procedure only. Table V showed that although RSD of each analyte was different between CAP-50 and EOB+CAP-50, values of Fcal, the experimental values of F-test between the two groups, were all less than the critical value of F0.05, 5, 5=5.05. It suggested that the differences of precision between the two groups were negligible and that the isolation procedure did not contribute significantly to the experimental errors. Therefore, the precision of isolation method for impurity test was, at least, within that of the HPLC analysis.

TABLE IV. LINEARITY OF ASSAY AND IMPURITY TEST (N = 4)

No.	Trendline Equation[a]	R^2	Slope RSD (%)
1	*y = 29 x*	0.991	2.04
2	*y = 225 x*	0.997	0.40
3	*y = 94 x*	0.993	0.70
4	*y = 129 x*	0.993	0.76
5	*y = 73 x*	0.997	0.75
6	*y = 14 x*	0.994	4.37
7	*y = 17 x*	0.991	3.37
Assay	*y = 1298 x*	0.9993	0.70

[a]: set intercept = 0

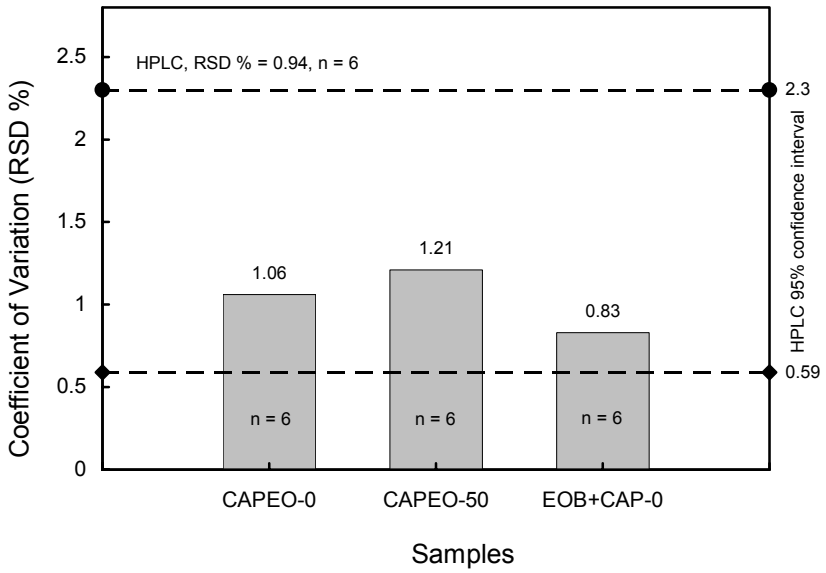

FIG. 2. Precision of assay test described by relative standard deviation (RSD). CAPEO-0 and CAPEO-50 represented chloramphenicol eye ointment non-irradiated and irradiated at 50 kGy, BOE+CAP-0 represented eye ointment base spiked with non-irradiated chloramphenicol powder.

TABLE V. PRECISION AND RECOVERY OF THE IMPURITY TEST (N = 6)

No.	RT[a]	CAP-50			EOB + CAP-50			*F* test	Recovery *t* test	
	min	Response[b]	SD	RSD%	Response[b]	SD	RSD%	F_{cal}	%	t_{cal}
1	3.1	25452	396	1.56	25880	610	2.36	2.37	101.7	1.44
2	7.2	16203	303	1.87	16404	149	0.91	4.15	101.2	1.46
3	13.2	8348	131	1.57	8385	96	1.14	1.85	100.4	1.57
4	14.1	25630	492	1.92	25162	286	1.14	2.95	98.2	2.02
5	15.2	7279	148	2.03	7345	106	1.44	1.93	100.9	0.89
6	17.2	3277	55	1.68	3256	67	2.06	1.52	99.4	0.60
7	23.0	1929	103	5.34	1956	65	3.32	2.50	101.4	0.54

[a] Retention Time; [b] Mean response of the impurity peaks from six replicates

3.4. Accuracy

For assay test, the accuracy of the method was evaluated by recovery and t-test from six replicates of spiked samples (EOB+CAP-0) at target concentration (Table VI). The recovery of CAP from spiked sample was 99.2%. Furthermore, the experiment value of t-test (tcal) between CAP-0 and EOB+CAP-0 was 1.21, less than the critical value of t0.05/2, 10 =2.23, indicating that there were no differences of analytical accuracy between EOB+CAP-0 and CAP-0 by the present method.

TABLE VI. RECOVERY OF THE ASSAY TEST (N = 6)

Method	CAP-0			EOB + CAP-0			Recovery	*t* test
	Response[a]	SD	RSD%	Response[a]	SD	RSD%	%	t_{cal}
Present	342698	4412	1.29	340101	2839	0.83	99.2	1.21
USP	344870	3876	1.12	313394	3949	1.26	90.9	13.93

[a]: response of chloramphenicol from six replicates

In contrast, the recovery was 90.9% and tcal equaled 13.9 by the method of USP (Table VI), which was far greater than the critical value. The USP method certainly gave different measured contents of CAP between CAP-0 solution and the spiked sample solution. It significantly undervalued the measured CAP content in the eye ointment, possibly due to the presence of white precipitates. However, proper analysis resulted by the USP method from different calibration curves could not be ruled out.

The results of t-test and recovery in Table V demonstrated that the current method was also accurate for impurity test. The t-test was performed to measure the closeness of analytical agreement between CAP-50 (going through only the HPLC procedure) and spiking sample EOB+CAP-50 (going through both the isolation and the HPLC procedures). Every experimental value of t-test (tcal) was less than critical value t0.05/2, 10 = 2.23, indicating that there were no significant differences in the measured impurity contents between the two groups. Thus, each impurity was isolated and analysed accurately.

3.5. Characterization of the Isolation Process

Necessity and Validation of Heating

It was found that some components of the eye ointment base could not be fully dissolved in both hydrophilic and hydrophobic solvents without heating. The insoluble residues left in the final solution not only needed to be filtered, but might also cause residue encapsulation or adsorption of the target compounds, which resulted in poor recoveries. Heating the n-hexane suspension at 45 °C made the residues easily dissolved, and improved the recoveries successfully (Fig. 3).

However, heating treatment rose immediately the question whether or not chloramphenicol was still stable, as it was subject to both thermal and photochemical degradation. In order to check the validation of this treatment, the spiked samples (EOB+CAP-0) were dissolved in 10 mL n-hexane, and heated in water bath at 45°C for different time intervals, then following the same sample preparation procedures.

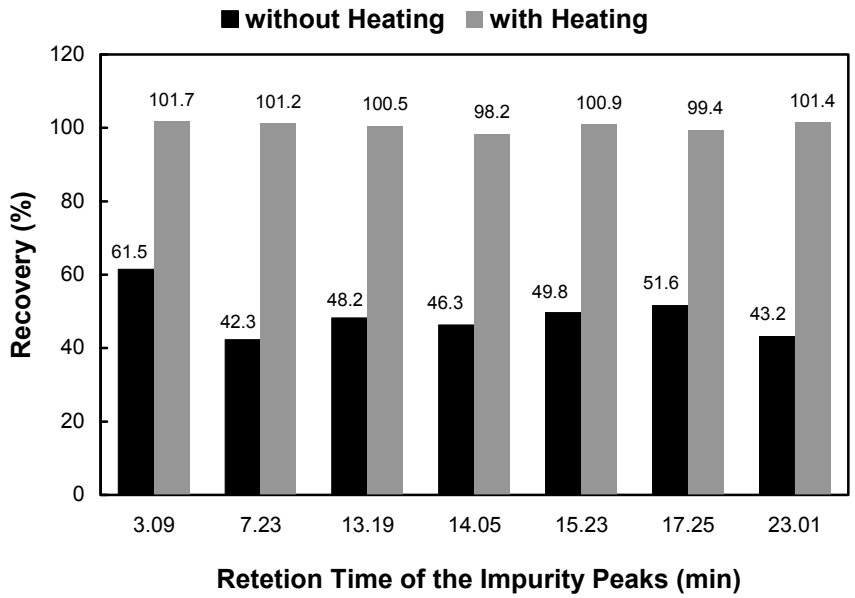

FIG. 3. Necessity of heating during sample preparation. The sample was treated with heating in 45 °C water bath and without heating at room temperature.

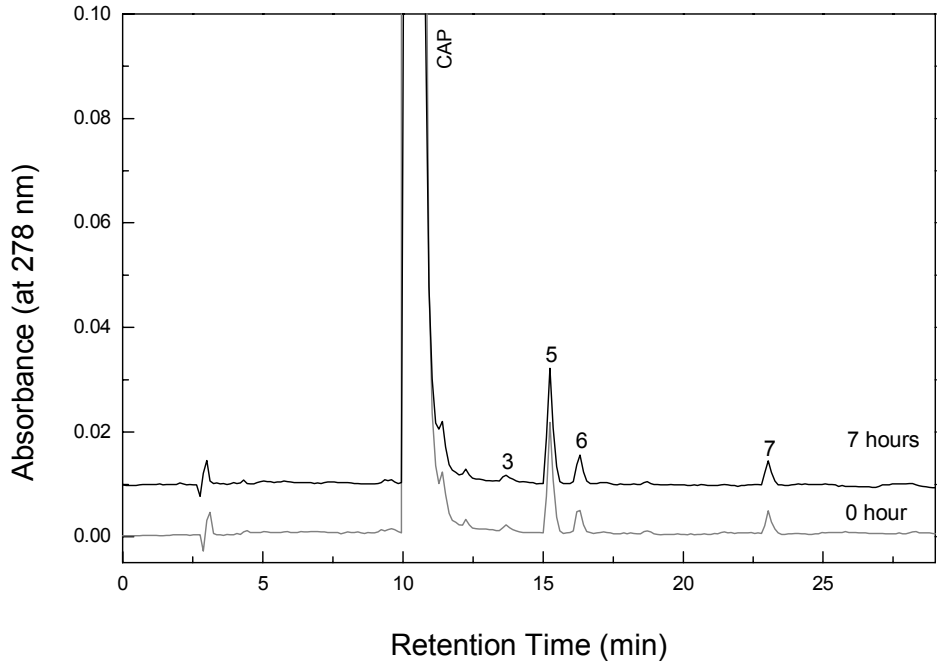

FIG. 4. Evaluation of thermal stability of CAP at 45 °C

Fig. 4 showed that no new compound was formed even after 7 h of heating treatment, and quantities of the original CAP and impurities had no visible variation as well. It could be concluded that chloramphenicol kept its thermal stability at 45°C, and the present heating treatment was valid.

Precipitates during Sample Preparation in USP

Methanol extraction was employed to extract CAP for assay test in USP. Severe white precipitates were formed in the final solution when the sample was suspended to the mobile phase of HPLC. To identify the precipitates, eye ointment base was dissolved and extracted according to the sample preparation procedures of USP. The extract solution was analysed by gas chromatography.

Fig. 5 showed that the extract solution included mainly 1-dodecanol, 1-tetracanol, 1-hexadecanol and 1-octadecanol (identification of the other smaller peaks will be reported in Chapter 6). Those compounds were extracted together with CAP and its radiolytic degradation products by the USP method, as they were soluble in methanol. However, they were insoluble in the mobile phase of HPLC for assay test of USP (the mixture solution of water, methanol and glacial acid), and presented as white precipitate. The mixture of 1-hexadecanol and 1-octadecanol was the well-known ingredient of eye ointment base and functioned as emollient and emulsifying. In the present isolation method, these compounds were soluble in n-hexane and thus were extracted into hydrophobic part.

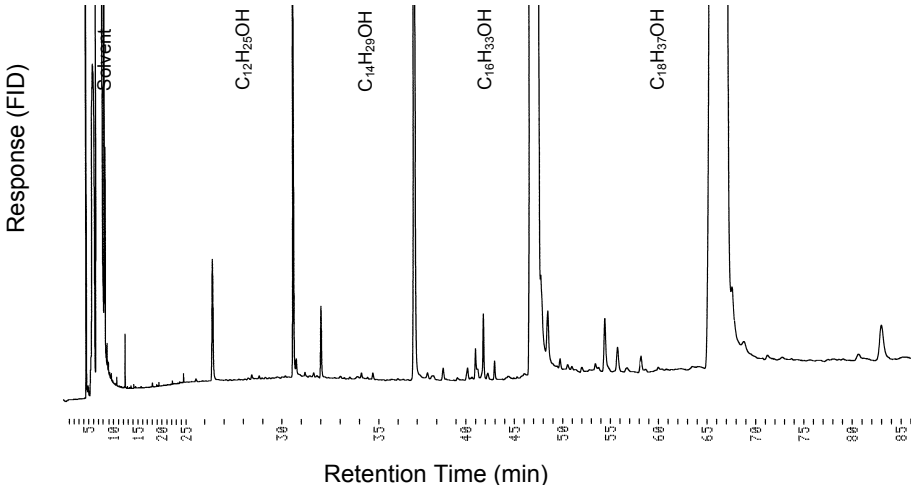

FIG. 5. Gas chromatogram of the methanol extracts of petrolatum eye ointment base.

Determination of Irradiated CAP

According to the report by Hangay et al., irradiated CAP did not show measurable changes either in pure powder state or in eye ointment after irradiation of 50 kGy dose. The present result, determined by HPLC, showed in contrast that CAP in eye ointment degraded significantly after irradiation (Fig. 1). It was noted that UV-spectroscopy method was employed by Hangay et al., and the radiolytic degradation products were not identified in their studies. The influence of impurities on the assay test results was therefore, not clarified.

The three dimensional chromatogram (Fig. 6) from HPLC diode array detector in the present study illustrated that impurities from CAPEO-50 also contributed to the UV absorbance almost at the same wavelength of maximum absorbance of CAP. Positive experimental errors were thus unavoidable. The argument was further demonstrated when the assay test results were compared. The UV-spectroscopy method according to BP gave a positive error compared to that of the HPLC method in Table VII. Therefore, the UV-spectroscopy method was unsuitable for assay determination of irradiated chloramphenicol products.

TABLE VII. ASSAY TESTS OF CAPEO-50 WITH DIFFERENT METHODS (N = 6), CAPEO-0 WAS SET AS 100%.

Sample	HPLC method		UV-spectroscope[a]	
	RSD (%)	Content (%)	RSD (%)	Content (%)
CAPEO-0	1.06	100	0.98	100
CAPEO-50	1.21	88.9	0.95	94.9

[a]: according to the method of the British Pharmacopoeia for assay test (at 278 nm)

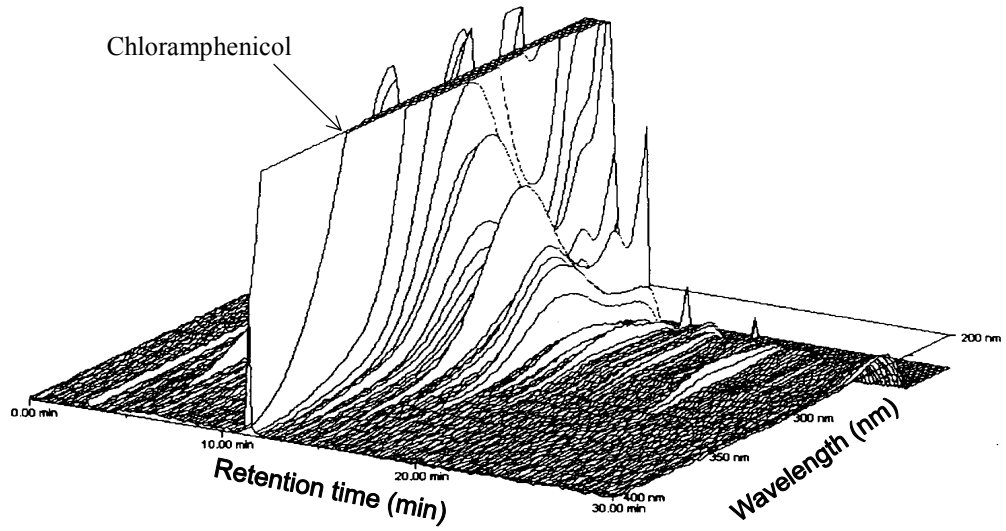

FIG. 6. Three dimensional HPLC chromatogram of chloramphenicol and impurities by diode array detector.

4. CONCLUSION

The present methods of isolation and determination of assay and impurity in CAP eye ointment were accurate, precise and reliable, and keeping the sample solution at 45°C during sample preparation was key to ensure a satisfactory isolation. It described for the first time a method to determine impurities in irradiated eye ointment products of chloramphenicol. In addition to simplified manipulation and low solvent consumption, the method isolated CAP and the impurities as dry residues, which ensured more flexibility for further determination.

The sample preparation methods of USP and BP were certainly not suitable for impurity determination of CAP eye ointment products, due to unsure exhausted extraction and the lean concentration in the resulting solution. Furthermore, methanol extraction of ointment products by USP was involved in problems with precipitates, which encapsulated the target compounds and undermined experimental results. The UV spectroscopy method in BP certainly was not able to exclude the absorbance contributions from the CAP degradation products, which resulted in positive errors in the assay test of irradiated chloramphenicol eye ointment products. HPLC was clearly a better choice for the determination of assay and impurities of irradiated chloramphenicol eye ointment.

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