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THIN-LAYER CHROMATOGRAPHY OF NUCLEIC ACID BASES, NUCLEOSIDES, NUCLEOTIDES AND RELATED COMPOUNDS

IX. QUANTITATIVE ANALYSIS BY *IN SITU* REFLECTANCE SPECTROSCOPY*

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SUMMARY

Five nucleo derivatives have been chosen for quantitative *in situ* determination by reflectance spectroscopy after two-dimensional separation from a complex mixture by thin-layer chromatography on cellulose. The use of an internal standard to compensate for fluctuations in the chromatographic procedure resulted in an improvement in the reproducibility of data by about 3–4% relative standard deviation. Average accuracies obtained with the method in the analysis of an artificial mixture ranged between 5.1 and 4.0 %. The use of a linear calibration curve of substance to standard ratio plotted *versus* square root of concentration proved most advantageous. The use of one set of calibration curves for the analysis of mixtures on various days gave an average error of about 16% but was tremendously timesaving. The method was successfully applied to the analysis of nucleo derivatives in an extract of cartilage red bone marrow.

INTRODUCTION

Reports on the reflectance spectroscopic evaluation of nucleo derivatives have been previously communicated[1–4]. Time studies revealed no significant changes, either with regard to peak shift or intensity of maxima, over a 24-h period[3]. Shifts of maxima over an analytically useful concentration range were ±2 nm (*cf.* ref. 3).

In view of these facts, it was decided to work out a quantitative *in situ* reflectance spectroscopic method for some nucleo derivatives with the use of the Zeiss

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chromatogram spectrophotometer. The method was to be applied to the determination of nucleo derivatives in biological materials.

EXPERIMENTAL

Chromatographically pure hypoxanthine, uridine, uracil, inosine and thymine have been used for the preparation of stock solutions in 0.04% v/w NaOH. In many cases adenine has been added as internal standard. The plates were coated with Cellulose MN-300 (Macherey, Nagel & Co., Düren, G.F.R.), purified according to ref. 5. All solvents used were of reagent grade.

Samples and standards were applied with 5-μl capillaries (Microcaps, Drummond Scientific Co. Ltd., Broomall, Pa., U.S.A.). Two-dimensional chromatography was carried out according to a previously described method[5]. The spots were viewed under an UV lamp (Camag Ltd., Muttenz, Switzerland) at 254 nm and marked on the backside of the plate with a grease pencil. Reflectance measurements were made with the Zeiss chromatogram spectrophotometer (Zeiss, Oberkochen, G.F.R.). The chromatographic peaks were evaluated by planimetry with a planimeter manufactured by Ott Ltd., Kempten, Bavaria, G.F.R.

RESULTS AND DISCUSSION

Calibration curves

The concentration–reflectance relationships of the 16 nucleo derivatives qualitatively investigated in the previous communication[3] have been studied in this proj-

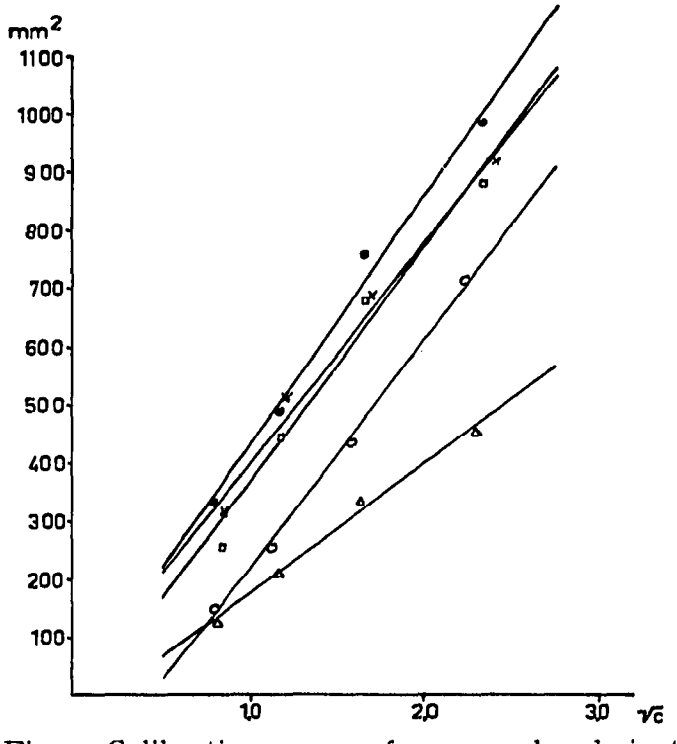

Fig. 1. Calibration curves of some nucleo derivatives determined by *in situ* reflectance spectroscopy on cellulose layers. ●, Hypoxanthine; ×, thymine; □, xanthine; △, nicotinamide; ○, AMP-3′.

ect. Fairly linear calibration curves were obtained with peak areas plotted *versus* square root of concentration ($\sqrt{c}$). A few examples of these calibration plots are given in Fig. 1. The curves have been computed statistically and correlation coefficients ranging between 0.980 and 0.994 have been obtained.

Internal standards

KLAUS[6] has introduced the use of an internal standard in one-dimensional thin-layer chromatography of bands, to compensate for fluctuations inherent in the chromatographic procedure. Such an internal standard would be even more desirable in two-dimensional chromatography and the advantage of using an internal standard for the problem at hand was clearly demonstrated with hypoxanthine (Table I). Three micrograms of uracil and adenine were added as internal standards to 3 μg of hypoxanthine. Chromatography was carried out in the usual way once one in a set of 6 and once of 12 analyzed on the Zeiss instrument. Improvements in reproducibility between 3 and 4 % relative standard deviation were observed with the use of both uracil and adenine as internal standards. Since uracil is itself a component of the biological samples of interest, it was decided to use adenine which is not present and does not interfere with any of the naturally occurring nucleo derivatives on the chromatogram.

TABLE I

A COMPARISON OF THE REPRODUCIBILITY OF DATA WITH AND WITHOUT THE USE OF INTERNAL STANDARDS

	Hypoxanthine	*Uracil*	*Adenine*	*Hypoxanthine/uracil*	*Hypoxanthine/adenine*
Mean and standard deviation[a] ($n = 6$)	752 ± 69	790 ± 65	780 ± 75	0.96 ± 0.069	0.96 ± 0.05
% Rel. st. dev.	9.1	8.3	9.6	6.3	5.1
Mean and standard deviation ($n = 12$)	700 ± 70	760 ± 81	740 ± 60	0.924 ± 0.066	0.948 ± 0.065
% Rel. st. dev.	10.0	10.7	8.1	7.1	6.9

[a] St. dev. $= s = \sqrt{\frac{\Sigma(M - \bar{M})^2}{(n-1)}}$.

Analysis of a synthetic mixture

Chromatographic separation has been carried out as usual[5]. The chromatogram, as well as the corresponding chromatographic peaks, are shown in Fig. 2. (Arrows mark the scan direction.) For quantitative work, the peak evaluation method also shown in Fig. 2 proved to be the best of a number of techniques tested, particularly for not completely resolved double peaks.

Calibration curves of ratios (substance/adenine) plotted *versus* concentration showed the usual bent shape (Fig. 3). Reasonably linear curves were obtained, with the origin generally differing somewhat from zero, if the ratios were plotted *versus* the square root of concentration (Fig. 3). Both calibration curves were used for evaluation purpose. The linear plots gave generally somewhat better results. The results of this analysis are presented in Table II. The total time per analysis is two days.

This includes chromatographic separation on one day and evaluation of data by the internal standard method on the second. Actual working time for one technician is about

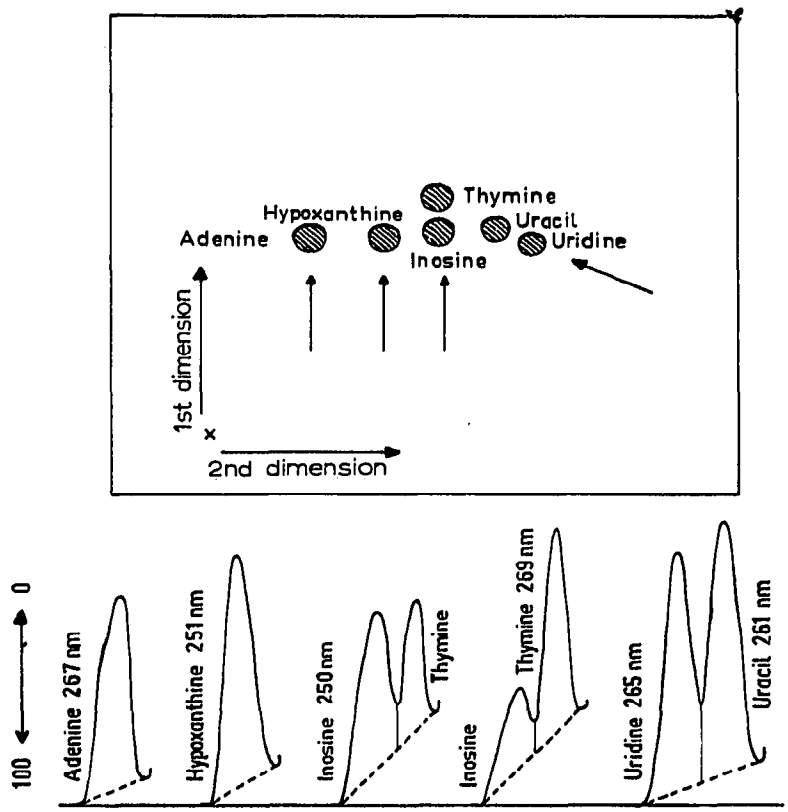

Fig. 2. Thin-layer chromatogram and corresponding chromatographic peaks. Scanning speed, 7.5 cm/min; recorder speed, 8 cm/min. Arrows mark the direction of scan.

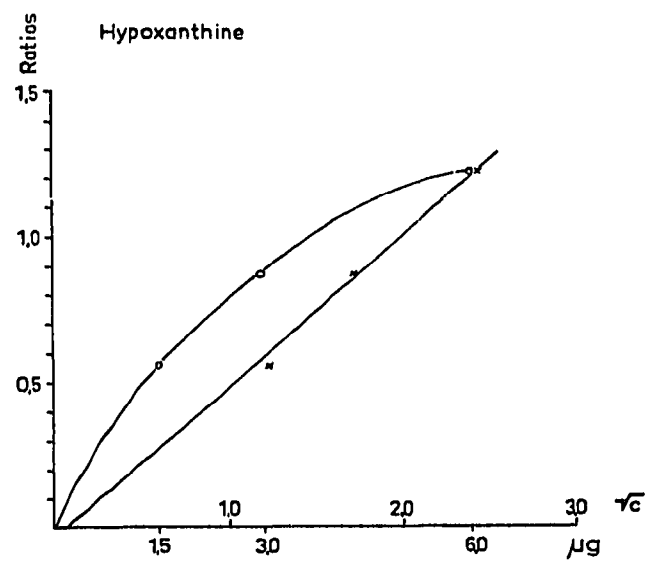

Fig. 3. Calibration curves of peak area ratios hypoxanthine/adenine plotted *versus* concentration (○—○) and *versus* square root of concentration (×—×) of hypoxanthine.

TABLE II

ANALYSIS OF A KNOWN ARTIFICIAL MIXTURE

Compound	*Amount present* (μg/spot)	*Amount found*[a]		*Percent deviation*	
		Conc.	$\sqrt{c}$	*Conc.*	$\sqrt{c}$
Hypoxanthine	4.12	4.56	4.26	10.7%	3.4%
Inosine	3.90	4.01	4.04	2.8	3.6
Thymine	4.35	4.06	4.11	6.7	5.5
Uridine	3.83	3.80	3.56	0.7	7.0
Uracil	3.60	3.43	3.65	4.7	1.4
			Average deviation	5.12%	4.02%

[a] Average of three independent analyses.

TABLE III

THE USE OF ONE SET OF CALIBRATION CURVES FOR THE ANALYSIS OF MIXTURES ON VARIOUS DAYS

Compound	*Amount present* (μg)	*Mixture*							
		1		*3*		*1*		*2*	
		Calibration curves set							
		2		*3*		*3*		*3*	
		Amount found		*Amount found*		*Amount found*		*Amount found*	
		(μg) $\sqrt{c}$	*Conc.*	(μg) $\sqrt{c}$	*Conc.*	(μg) $\sqrt{c}$	*Conc.*	(μg) $\sqrt{c}$	*Conc.*
Hypoxanthine	4.12	5.48	5.3	4.97	4.75	5.06	4.8	4.24	4.50
Percent error		33.0	28.6	20.6	15.3	22.8	16.5	2.9	9.2
Inosine	3.9	—	—	4.45	4.6	—	—	3.03	3.00
Percent error		—	—	14.1	18.0	—	—	22.3	23.1
Thymine	4.35	5.15	5.25	5.15	5.25	3.8	3.63	3.28	3.18
Percent error		18.4	20.7	18.4	20.7	12.6	16.5	24.6	26.9
Uridine	3.83	—	—	4.00	4.15	—	—	2.82	2.85
Percent error		—	—	4.4	8.3	—	—	26.4	25.6
Uracil	3.6	3.6	3.45	2.72	2.68	3.76	3.63	3.53	3.45
Percent error		0.2	4.2	24.4	25.6	4.4	0.8	1.9	4.2
Average percent error for one analysis		17.1	17.8	16.4	17.6	13.3	11.3	15.6	17.8
						Total average percent error		15.6	16.2

6–8 h. Nine standards of three different concentrations and three samples were chromatographed on twelve plates in the same chromatographic tank. Number of plates and separation time would remain the same no matter how many components are being determined in the mixture, which makes the method timewise even more attractive.

ABLE IV

ESULTS OF ANALYSIS OF THREE BATCHES OF CARTILAGE EXTRACT (RUMALON)

ompound	Batch 3 (MK 4087)				Batch 2[a] (MK 4072)		Batch 1[a] (MK 4048)	
	$\sqrt{c}$ (μg)		Conc. (μg)		$\sqrt{c}$ (μg)	Conc. (μg)	$\sqrt{c}$ (μg)	Conc. (μg)
	St. dev.[b]	% St. dev.	St. dev	% St. dev.				
[ypoxanthine	3.63 ± 0.36	10	3.62 ± 0.42	11.5	1.35	1.20	2.82	2.75
nosine	0.51 ± 0.26	51	0.46 ± 0.17	37	0.26	0.23	0.25	0.28
[ridine	1.23 ± 0.23	18.7	1.24 ± 0.29	23.4	0.96	0.95	0.49	0.45
[racil	5.18 ± 0.36	6.9	4.77 ± 0.45	9.5	1.04	1.10	1.80	1.77

[a] Results are obtained from one analysis for each batch.
[b] Standard deviations are calculated from six analyses.

The use of one set of calibration curves for the determination of mixtures analyzed on different days was also investigated (Table III). An average percent error of around 16% was found compared to around 4 and 5% error with standards chromatographed simultaneously with every determination (Table II). The timesaving factor is however so enormous that the latter method may have some merit for certain applications.

Analysis of biological material

Cartilage red bone marrow extract (Rumalon, Robapharm Ltd., Basle, Switzerland) was examined for these components. The same chromatographic procedure was used[5], and a complete spotchart and data on a preliminary investigation of this system were given earlier[1]. After freeze-drying the various batches, 5:1 dilutions were made and analyzed similar to the artificial mixture. The reproducibility of hypoxanthine in Rumalon was checked. A relative standard deviation of 605 mm^2 ± 37 or 6.1% was found. This is in the order of magnitude of reproducibilities observed with the artificial mixture.

Results obtained from three batches of Rumalon are presented in Table IV. The high percentage of relative standard deviation for inosine is due to the low concentration of this component, which at a dilution ratio of 5:1 approaches a detection limit. Extracts of higher concentration are hard to handle. From Table IV it can be seen that the differences in concentration of hypoxanthine and uracil in the three batches are significant. Differences of inosine are not significant and differences of uridine between batches number 1 and 2 are barely significant. The use of 4 times 2 standards of 4 different concentrations for the calibration curve proved somewhat more advantageous than the use of 3 times 3 standards of only 3 different concentrations.

CONCLUSIONS

Reflectance spectroscopy can be used as a means of quantitative determination of nucleo derivatives in biological systems. Even though the method has been applied only to a small group of such compounds, it could well be extended to all the nucleo derivatives tested earlier and essentially to all stable and ultraviolet-active organic systems. In the case of Rumalon, the method can be adopted as a semiroutine analysis for periodic checks of the nucleo derivative content of the various extraction batches, prior to entering the pharmaceutical processing stage.

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