Journal of Chromatography, 93 (1974) 399–404

CHROM. 7347

SEMICONDUCTOGRAPHIC DETERMINATION OF LABELLED SUBSTANCES IN THIN-LAYER CHROMATOGRAPHY

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(Received January 2nd, 1974)

SUMMARY

A method is presented for the radioactive detection of β-nuclide-labelled substances in thin-layer chromatography. The method is based on a scanning device with a semiconductor detector; the detection proceeds in the laboratory atmosphere and at room temperature. The measurement and recording may be programmed on a computer. Conditions are considered for ^{3}H- and ^{14}C-detection and also for the detection of simultaneously present ^{14}C and ^{35}S, after the chromatographic separation of nucleic acid components.

INTRODUCTION

The chromatographic separation of radionuclide-labelled substances on a thin layer has been widely used in the preparation and storage of these substances and also in their application in tracer experiments. In the radioactive detection of organic compounds, low-energy β-nuclides (^{3}H, ^{14}C, ^{35}S) are most frequently involved. The methods for the determination of these nuclides in thin-layer chromatography (TLC) can, in principle, be divided into two groups. The chromatographic layer remains intact in the first group of methods while in the second group some modifications of the chromatographic system are made prior to the radioactive detection. The first group comprises the gas flow counter with a moving device[1], autoradiography[2] and the spark counter[3], while in the second group the scintillation technique is most frequently used. The scintillator is introduced into the chromatographic layer and detection is then performed either (a) on a photographic emulsion[2], (b) by means of a scanning device with a photomultiplier[4], (c) the carrier with the chromatographic layer is cut into pieces which are measured separately in vials containing a liquid scintillator[5], or (d) the appropriate parts of the chromatographic layer are scraped off and measured in a liquid scintillation spectrometer[6].

In this paper, we report a non-destructive method based on semiconductography[7] for the determination of β-nuclide-labelled substances in TLC. The measurement is performed at room temperature in the usual laboratory atmosphere using the movement of the chromatographic layer under a silicon barrier detector[8]. The two-dimensional movement may be either semi-manual or may be programmed on

a computer. The automated record of the counting rates at each position measured may be performed either numerically (counts in a certain time interval) or graphically on a 1:1 scale (each order of magnitude of the counting rate measured is recorded in a different colour). The high-energy resolution of the semiconductor detector makes it possible to distinguish by means of amplitude analysis at each position the particular β-nuclides that are simultaneously present[9]. In this paper, conditions are examined for the simultaneous detection of ^{14}C and ^{35}S in thin layers; none of the existing methods permits such a simultaneous detection. The method has been used with success[10], for example, in the preparation of thio derivatives of nucleic acid bases simultaneously labelled with ^{14}C and ^{35}S.

EXPERIMENTAL

Materials

Radioactive compounds such as [^{14}C]adenine, [^{14}C]guanine, [^{14}C]cytosine, [^{14}C]uracil, [^{3}H]uridine and [^{3}H]uridine 5'-monophosphate were obtained from the Institute for Research, Production and Application of Radioisotopes, Prague, Czechoslovakia; 4-thio-[^{14}C]uracil, 4-[^{35}S]thiouracil and 4-[^{35}S]thio-[^{14}C]uracil were prepared according to Šeda *et al.*[10]. The specific activity of commercially available substances was verified by comparison with ^{3}H- and ^{14}C-labelled standards in an internal gas counter[11]. The TLC of ^{14}C-labelled compounds was performed on ready-for-use Silufol UV_{254} silica gel plates (Sklárny Kavalier, Czechoslovakia). The TLC of ^{3}H-labelled compounds and also ^{14}C-, ^{35}S- and ^{14}C,^{35}S-labelled 4-thiouracil was carried out on ready-for-use MN-Polygram Cell 300 plates (Macherey, Nagel & Co., Düren, G.F.R.).

Solvent mixtures

TLC was performed with the use of the following freshly prepared solvent mixtures: (1) 1-butanol–5.2% aqueous ammonia (86:14); (2) 1-butanol–glacial acetic acid–water (10:1:3); and (3) 2-propanol–concentrated aqueous ammonia–water (7:1:2).

Chromatography

The ^{14}C-labelled samples (1 μl each) of adenine (6070 dpm per 0.8 μg/μl), cytosine (15870 dpm per 1.43 μg/μl), uracil (1910 dpm per 1.4 μg/μl) and guanine (7520 dpm per 0.5 μg/μl) were applied to the starting line (placed 1 cm above the lower edge of the plate; length 0.3 cm) of Silufol UV_{254} (8.5×8.5 cm) and chromatographed by the ascending technique in solvent mixture 1. When the solvent front had migrated 7.0 cm from the starting line, the plate was removed from the tank and air-dried at room temperature. The dry plate was rotated through 90° and then chromatographed in solvent mixture 2.

The ^{3}H-labelled samples (10 μl each) of uridine (0.226 μCi per 0.24 μg/μl) and uridine 5'-monophosphate (0.247 μCi per 0.36 μg/μl) were applied to the starting line (placed 1 cm above the lower edge of the plate; length 0.4 cm) of a MN-Polygram Cel 300 plate (10.0×8.5 cm) and chromatographed by the ascending technique in solvent mixture 3 until the solvent front had migrated 8.0 cm.

Labelled thiouracils, namely, 4-thio-[^{14}C]uracil (0.39 μg per 0.15 μCi/μl; 1 μl), 4-[^{35}S]thio-[^{14}C]uracil (0.21 μg per 0.42 μCi/μl; 6 μl) and 4-[^{35}S]thiouracil

(0.18 μg per 0.50 μCi/μl; 5 μl) were chromatographed (length of starting line 0.5 cm) in a similar manner to the tritiated samples using solvent mixture 2.

Measurement of radioactivity

The measuring apparatus is shown schematically in Fig. 1. The chromatogram is attached to a moving table, the movement of which in the system of orthogonal coordinates may be either semi-manual or may be programmed by a computer[7]. The step length in each direction can be adjusted in 1-mm steps in the range from 1 to 7 mm; in the present work, the 1-mm step was used. A measurement in a particular position may last from 10 sec to 2½ h; depending on the radioactivity of the substance present, different parts of the chromatogram may be measured for a different period of time (see Fig. 2). The measurement in each position may be

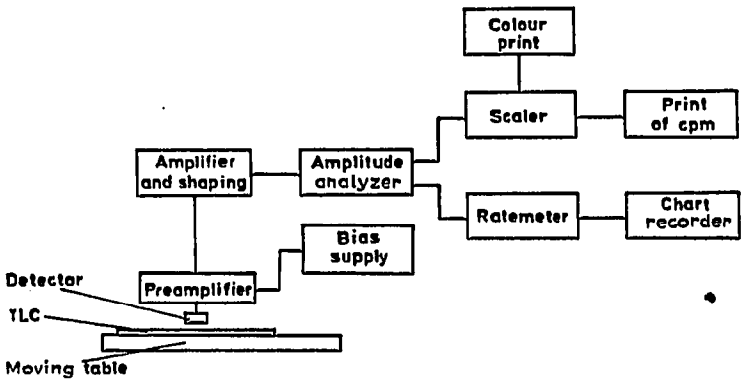

Fig. 1. Scheme of the apparatus for the semiconductographic detection of labelled substances in chromatographic thin layers.

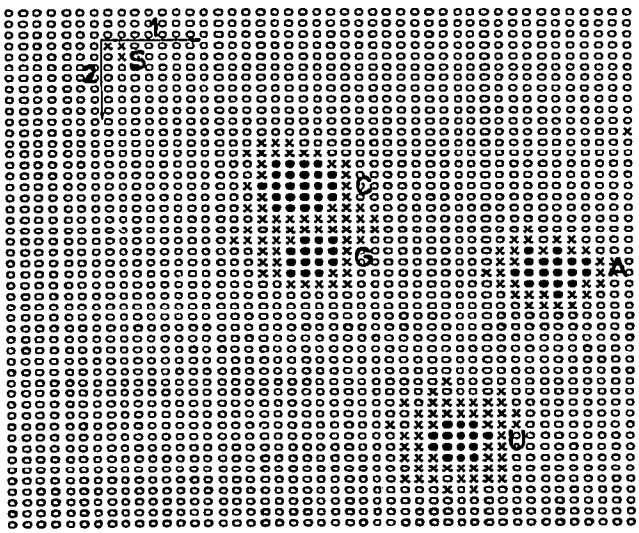

Fig. 2. TLC of ^{14}C-labelled adenine (A), guanine (G), cytosine (C) and uracil (U). Time of measurement: 1 min (A, G, C); 5 min (U). S=start. ○, background; ×, 1–9 and ●, 10–99 counts in the chosen time interval.

repeated from two to nine times. At a small distance (usually 0.5 mm) above the chromatographic layer and in a constant position is placed the silicon barrier detector with an input electrode 1 mm in diameter[8].

After amplification, voltage impulses from the detector are selected by amplitude analysis according to the energy range of the β-spectrum emitted by the radionuclide being measured. In the determination of single radionuclides (^{3}H or ^{14}C), a single-channel device is sufficient; in this device, the counting window levels are defined by the discrimination level required to exclude noise from the record (9 keV) and by the discrimination level corresponding to the maximum energy of the β-spectrum (18 and 156 keV, respectively). When ^{14}C and ^{35}S are simultaneously present at the position being measured, a two-channel measuring system is used. In the "total" channel (9–170 keV), the lower level is defined by the requirement to exclude noise while the upper level is determined by the maximum energy of the emitted β-spectra (167 keV) with respect to the energy resolution of the apparatus (the FWHM value is approximately 7 keV). In the "sulphur" channel, the window is set in such a manner as to ensure that only the particles emitted by ^{35}S are recorded (for details, see Table I). The activity of the radionuclides is calculated from the data obtained in both channels in an analogous manner to the two-channel measurements in a liquid scintillation spectrometer[12].

TABLE I

COUNTING RATES IN VARIOUS CHANNELS

Thin-layer chromatography on MN-Polygram Cel 300.

Channel (keV)	*Counting rate (cpm)*	
	^{14}C	^{35}S
18–170	4334 ± 4	9915 ± 9
156–170*	0.10 ± 0.03	4.50 ± 0.05
160–170	0.00	2.01 ± 0.04

* Background, 0.02 ± 0.01 cpm.

The counting rate in a particular channel is recorded for each measured position either numerically by means of a printer (counts in a certain time interval) or by coloured dots. The latter record is produced in six colours on a 1 : 1 scale with respect to the chromatogram. One colour corresponds to the background measurement while the remaining five colours refer to the different levels of the detected radioactivity (units, tens, hundreds, thousands and more impulses in the particular time of the measurement).

RESULTS AND DISCUSSION

The sensitivity of detection of ^{14}C was determined for the separation of [^{14}C]-adenine, [^{14}C]guanine, [^{14}C]cytosine and [^{14}C]uracil (Fig. 2). In the 9–156 keV channel, the background was 0.17 ± 0.02 cpm. The detection efficiency was expressed as the arithmetic mean of the values obtained for all four substances. For each sub-

stance, the efficiency was calculated as the ratio of the sum of the counting rates determined by measurements at all positions of the spot to the sum of the number of disintegrations in the same time interval. The number of disintegrations was defined by the specific activity and the amount of the chromatographed substance. The final value of this detection efficiency was 5.6% in the thin layer measured. The present figure of merit [(efficiency)^2/background] is thus comparable with that of the scanning device with a small- or low-background windowless flow counter.

The sensitivity of detection of ^{3}H for the separation of [^{3}H]uridine and [^{3}H]uridine 5'-monophosphate (Fig. 3) was determined in a similar manner. In the 9–18 keV channel, the background was 0.09 ± 0.01 cpm. On the layer applied, the average efficiency was $0.051 \pm 0.005\%$. In view of the attainable sensitivity of detection, the method is suitable for use with tritiated substances of a higher radioactivity, *e.g.*, in the preparation of labelled substances.

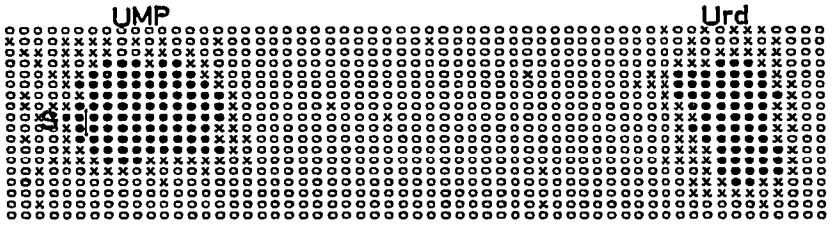

Fig. 3. TLC of [^{3}H]uridine (Urd) and [^{3}H]uridine 5'-monophosphate (UMP). Time of measurement: 5 min. S=start. ○, background; ×, 1–9 and ●, 10–99 counts in the chosen time interval.

The conclusion on the sensitivity of detection of ^{14}C also holds for ^{35}S because of the close resemblance of the energy spectra of emitted electrons. The counting channel settings for ^{35}S are analogous to those in the above examples, *i.e.*, 9–167 keV. The method can also frequently be used in the TLC of substances labelled with β-nuclides of a higher energy spectrum (*e.g.*, ^{32}P) owing to the sensitivity of detection[9] and the low self-absorption in the chromatographic layer. The lower discrimination level of the counting channel for these nuclides is defined (in a similar manner to low energy β-nuclides) by the requirement to exclude noise, while the upper level is determined by the depth of the depletion layer of the detector[9].

The conditions required for the simultaneous detection of ^{14}C and ^{35}S were examined using settings of the counting channel where only disintegrations of the ^{35}S radionuclide are recorded (Table I). It can be seen from the results obtained that the setting of the lower discrimination level of this "sulphur" channel equal to the maximum energy of the ^{14}C β-spectrum (156 keV) is suitable owing to the average energy resolution of the apparatus (7 keV) only in those instances when considerably more ^{35}S than ^{14}C is present. In the opposite case, the lower discrimination level of the "sulphur" channel must be as high as 160 keV so as to ensure that ^{14}C is excluded from the record. When such a channel setting is used, however, the detection efficiency of ^{35}S is lowered more than twice. The results were verified by measuring the radioactivity of ^{14}C and ^{35}S in 4-[^{35}S]thio-[^{14}C]uracil after the TLC of a mixture of 4-thio-[^{14}C]uracil and 4-[^{35}S]thiouracil. These results for the mixture were in accordance with the values calculated from the radioactivity of the singly labelled

starting substances within the limits of the statistical deviation as determined according to Tykva[12].

On the basis of our present and earlier[9,13,14] results, conditions may be found for the semiconductographic method in chromatographic thin layers, thus allowing the simultaneous determination of labelled substances in the case of some additional combinations such as $^{14}C+^{32}P$ or $^{3}H+^{14}C+^{32}P$.

Owing to the thin input window in the barrier detector used[8] it can be assumed that the present arrangement will make possible a further increase in the detection efficiency, especially by a decrease in the detector noise.

CONCLUSION

The semiconductographic method described for the radioactive determination of labelled substances in chromatographic thin layers makes possible the programmed and non-destructive detection in the usual laboratory atmosphere and at room temperature. The very low background enables a sensitivity of detection to be attained such as is required in work with labelled substances, *e.g.*, in the determination of ^{14}C or ^{35}S (and also ^{32}P) in tracer experiments; with ^{3}H, the method is suitable in the case of higher radioactivity, *e.g.*, in the preparation of labelled substances. The high energy resolution makes possible the simultaneous determination of β-nuclides in multi-labelled substances on a thin-layer chromatogram, *e.g.*, the resolution of ^{14}C and ^{35}S (none of the currently used TLC methods has so far made possible such a discrimination).

ACKNOWLEDGEMENTS

The work was carried out under contract 1019/R1/RB of the International Atomic Energy Agency, which provided the financial support. Thanks are due to Mr. V. Pánek for valuable technical assistance.

REFERENCES

1 F. Berthold, *Proc. Symp. Radioisotope Sample Meas. Tech. Med. Biol., 1965*, International Atomic Energy Agency, Vienna, 1965, p. 303.
2 K. Randerath, *Anal. Biochem.*, 34 (1970) 188.
3 B. R. Pullan, in E. J. Shellard (Editor), *Quantitative Paper and Thin Layer Chromatography*, Academic Press, London, 1968, Ch. 11.
4 V. M. Vdovjenko, V. N. Bobrova, A. V. Zharkov, O. A. Rysjev and V. V. Volina, *Radiokhimiya*, 13 (1971) 225.
5 I. Votruba, A. Holý and R. H. Wightman, *Biochim. Biophys. Acta*, 324 (1973) 14.
6 F. Snyder, in S. Rothchild (Editor), *Advan. Tracer Methodol.*, 2 (1965) 107.
7 R. Tykva, *Advances in Physical and Biological Radiation Detectors*, International Atomic Energy Agency, Vienna, 1971, p. 211.
8 R. Tykva, *Excerpta Med., Int. Congr. Ser.*, No. 301 (1973) 455.
9 R. Tykva and V. Pánek, *Radiochem. Radioanal. Lett.*, 14 (1973) 109.
10 J. Šeda, I. Votruba and R. Tykva, *J. Label. Compounds*, in press.
11 R. Tykva, *Int. J. Appl. Radiat. Isotop.*, 18 (1967) 45.
12 R. Tykva, in H. Simon (Editor), *Anwendung von Isotopen in der Organischen Chemie und Biochemie*, Vol. 2, Springer, Berlin, Heidelberg, New York, 1974, Ch. F.
13 R. Tykva and I. Votruba, *Anal. Biochem.*, 50 (1974) 18.
14 R. Tykva, R. Jelínek and M. Seifertová, *Experientia*, in press.