Journal of Chromatography, 531 (1990) 101–129
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5401

Review

Radiochromatography in pharmaceutical and biomedical analysis

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(First received November 23rd, 1989; revised manuscript received February 9th, 1990)

CONTENTS

1. INTRODUCTION

Since radioisotopes became available on a commercial scale in the late 1940s, they have become an indispensable tool for solving a wide variety of analytical problems. Pharmaceutical and biomedical analyses are especially dependent on the use of radiolabelled analytes because most of the studies deal with the determination of minute amounts of material in complex matrices. Radioisotopes then

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provide a way of selectively studying the distribution and metabolism of exogenous and endogenous material in biological matter. Radiolabelled compounds are further extensively used in the determination of enzyme activities and in radioimmunoassays. Obviously, most of these applications rely on the separation of the compounds in one or more stages of the experiment, with subsequent radioactivity counting. Accordingly, various techniques for radioactivity counting applied to chromatographic separations have been developed.

It is the aim of this review to summarize recent developments in radioactivity detection in high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary zone electrophoresis (CZE) and planar chromatographic techniques, in relation to pharmaceutical and biomedical analysis. The emphasis will be on the radioactivity counting of the common radioisotopes ^{3}H, ^{14}C and ^{32}P. Applications will be mentioned infrequently.

It is assumed that the reader is familiar with the general practice of radioactivity detection and the various types of chromatography. General information on radioactivity detection and on the use of radioisotopes in chemical analysis may be found in ref. 1. An excellent monograph on radiochromatographic methods was issued in 1978 [2]. More recent discussions on radiochromatographic methods can be found in refs. 3 (exclusively HPLC), 4 (HPLC and TLC, mostly concerned with γ-emitting radioisotopes), 5 (applications in radio-HPLC), 6, 7 and 8.

2 RADIO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

2.1. Low-energy β-emitting radioisotopes

In HPLC, three methods are in common use for the detection of low-energy β-emitters (^{3}H, ^{14}C, ^{35}S, ^{63}Ni), *i.e.* off-line liquid scintillation counting (SC), flow-through SC and flow-through heterogeneous SC. The relative advantages and disadvantages of these methods have been described [3] and will be summarized below.

2.1.1. Off-line liquid scintillation counting

In off-line liquid SC, successive eluate fractions of typically 0.1–1.5 ml are collected manually or automatically into counting vials. After the addition of liquid scintillator and vigourous mixing, these are placed into a sample trail and counted separately in a scintillation counter. Despite the disastrous influence of the fraction volume on the chromatographic integrity and the laborious procedures involved in sample preparation, sample counting and data handling, this technique is still in widespread use. This is not exclusively due to the easy access of most laboratories to off-line scintillation counters.

Some important advantages of this detection mode are (i) the freedom in selecting the counting time of the samples independently from the separation

process, (ii) the relative stability of the background count-rate, which is largely set by the instrument and its environment, and (iii) the possibility of suppressing the influence of short-lived chemiluminescence processes on the counting results simply by increasing the waiting time between mixing of the sample and sample counting. Obviously in flow-through counting modes this is more difficult to accomplish, and chemiluminescence has been reported to occur after alkaline or ethereal solvents have been mixed with dioxane-based cocktails [9].

The inherent drawback of liquid SC is that the analytes of interest are lost for further analysis unless some form of eluate splitting is used. A possible, although quite laborious approach to this problem may be found in the work of Devine and Milborrow [10], who studied the isolation of ^{14}C-labelled analytes from liquid scintillators on alumina or silica Sep-Pak cartridges. Androstenedione and carotene were used as test compounds. Quantitative recovery for these compounds from liquid scintillators could be obtained, provided the scintillator did not contain detergents.

Another disadvantage of off-line SC stems from the collection of the eluate fractions, which may become irreproducible at increasing water content in the mobile phase [11]. This effect was attributed to the increase in surface tension of the eluate droplets with increasing water content. Finally, counting efficiences of successive sample vials may be expected to fluctuate for eluate–scintillator mixtures that are prone to form two-phase systems on standing. It is interesting to note that this drawback is normally irrelevant in flow-through counting.

From the above considerations it can be argued that off-line SC is applicable in situations where a small number of well separated peaks are expected, containing only minor amounts of radioactivity, *e.g.* in radioenzyme assays [12–14]. As an example, Abeijon *et al.* [13] measured the enzyme activity of cytidine monophosphate-N-acetylneuraminic acid (CMP-NeuAc) synthetase, using ^{3}H-labelled neuraminic acid (NeuAc) as substrate and radio-HPLC for the separation of NeuAc and CMP-NeuAc. Fig. 1 shows that only two radioactive signals were detected, corresponding to the substrate and the product.

Recently, scintillator cocktails have been introduced that are based on alkylbenzyl-type solvents [15,16]. These solvents are less toxic and have higher flamepoints than the more common cocktails on toluene, xylene, pseudocumene or dioxane. The storage costs of the scintillator cocktails and additional safety measures in handling may thus be reduced substantially.

The performance of any type of scintillator cocktail can be judged from the "Figure of Merit", FM = $E^2/\mathrm{cpm_b}$, in which E is the counting efficiency and $\mathrm{cpm_b}$ the background count rate in counts per minute. In a comparative study on various scintillator cocktails for ^{3}H, dramatic differences in FM values (of over 200-fold) have been observed [16]. The best overall performance was obtained with a toluene-based cocktail, and it was concluded that selection of any of the aforementioned "drain-disposable" cocktails will result in a degradation of performance for unquenched to moderately quenched tritium samples, relative to the toluene reference standard cocktail [16].

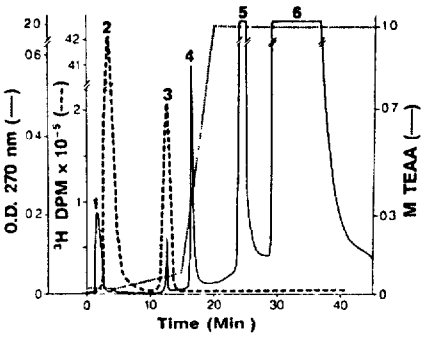

Fig 1 Radiochromatographic (HPLC) separation of CMP-NeuAc (peak 3) from substrates Column, SynChropak AX-100 (250 mm × 4 1 mm I D.), mobile phase, triethylamine acetate (TEAA), pH 7 0, gradient from 10 m*M* to 1 *M* TEAA After UV detection at 271 nm, samples of 2 ml of the effluent were collected The radioactivity of each fraction was determined following addition of 18 ml of liquid scintillator Peaks: 1 = cytosine, 2 = NeuAc (^{3}H substrate); 3 = CMP-NeuAc, 4 = CMP, 5 = CDP, 6= CTP (Reproduced with permission from ref. 13)

As an alternative to off-line liquid SC, Karmen *et al.* [17] developed a method for the collection of HPLC column eluates on filter paper. An automated microfraction collector was adapted to collect fractions of up to 0.30 ml into wells, pre-formed in a non-wetting fluorocarbon film. After partial evaporation, the spots are transferred to the filter paper by a vacuum technique, after which the radioactivity distribution on the paper is determined by conventional autoradiography. Exposure of 72 h allowed the determination of 5 dpm ^{14}C per spot. The linear dynamic range was found to depend on the exposure time, which is inherent to radioactivity detection by autoradiography.

2.1.2. Flow-through scintillation counting

During the past decade, flow-through SC has developed into a viable alternative to off-line SC. Flow-through devices are now available from at least six manufacturers (see Table 1) The systems normally include a liquid pump for post-column addition of liquid scintillator to the column eluate (optional), an eluate splitting device (optional), various types of flow cells at diferent cell volumes and software for data acquisition and data handling. All systems can be used in both the liquid and the heterogeneous mode.

2.1.2.1. Flow-through liquid scintillation counting. In most applications of radio-HPLC, reversed-phase conditions are used. Under these circumstances, a water-miscible liquid scintillator is preferred, which is continuously pumped to the aqueous column eluate via a small-bore mixing-tee just before entering the detector. In more demanding situations, *i.e.* the counting of ^{3}H-labelled analytes in eluates of high ionic strenght and high water content, a specially designed mixing device may improve the counting performances [18].

The counting efficiency can be affected by gradient elution, as shown by Ro-

TABLE 1

COMMERCIALLY AVAILABLE FLOW-THROUGH RADIOACTIVITY DETECTORS

Type(s)	Manufacturer
RAMONA radioactivity detectors	Isomess Isotopengeräte (Straubenhardt, F.R G.)
Beta-MAT	IN/US (Fairfield, NJ, U.S A.)
Betacord 1208	LKB Wallac (Turku, Finland)
Isoflo	Nuclear Enterprises (Edinburgh, U K)
Flo-One (IC, CR, CT, BD)	Radiomatic Instruments and Chemical Co. (Tampa, FL, U.S A)
LB 506 (A, B, D)	Berthold Labs (Wildbad, F.R G.)
Model 171/170	Beckman Instruments (Fullerton, CA, U.S A)

berts and Field [11]. In their set-up, ^{3}H efficiencies of 21, 13 and 22% were obtained for 0, 60 and 100% acetonitrile in the eluate, respectively. It is therefore important that a curve of the counting efficiency *versus* the gradient parameters can be recorded and stored in the computer memory for subsequent analysis.

Recently, a trend towards low (less than 5) scintillator/eluate mixing ratios has been apparent [19–21], partly because of improvements in the flow performance of new types of scintillator cocktail especially formulated for dynamic flow counting. The advantage of using lower mixing ratios lies in the correspondingly smaller volume of radioactive waste produced per chromatogram; the sensitivity may also be improved. This latter argument can be explained by considering the total flow-rate through the radioactivity detector which, self-evidently, decreases at decreasing mixing ratios. As a result, the mean residence time in the flow cell (*i.e.* the counting time) increases. Although at low mixing ratios the counting efficiency is not optimal, the sensitivity (*i.e.* the product of counting efficiency and mean residence time) may reach its maximum value. Fig. 2 illustrates this. Repetitive injections of ^{3}H- and ^{14}C-labelled proline and hydroxyproline were made at a mobile phase flow-rate of 0.6 ml/min. Flow-through liquid SC was performed in a 0.50-ml flow cell as a function of the liquid scintillator flow-rate. In Fig. 2a–c, the counting efficiency, the peak areas (in counts) and the number of theoretical plates are plotted, respectively. As can be seen, the peak areas are at maximum at a scintillator/eluate ratio of *ca.* 3.0. Unfortunately, in this example the band broadening increased rather rapidly at decreasing mixing ratios.

Band broadening in flow-through liquid SC can be suppressed effectively by gel or solvent segmentation of the column eluate, as demonstrated by Bakay [22] and Veltkamp *et al.* [19, 23–25], respectively. The principle published by Veltkamp *et al.* is based on the post-column extraction/segmentation of column eluates with liquid scintillator. To this end, a water-immiscible liquid scintillator is pumped continuously into the aqueous column eluate via a small-bore mixing-tee. As a result, a solvent-segmented stream consisting of alternating aqueous and organic segments is obtained, which is led through an extraction coil before enter-

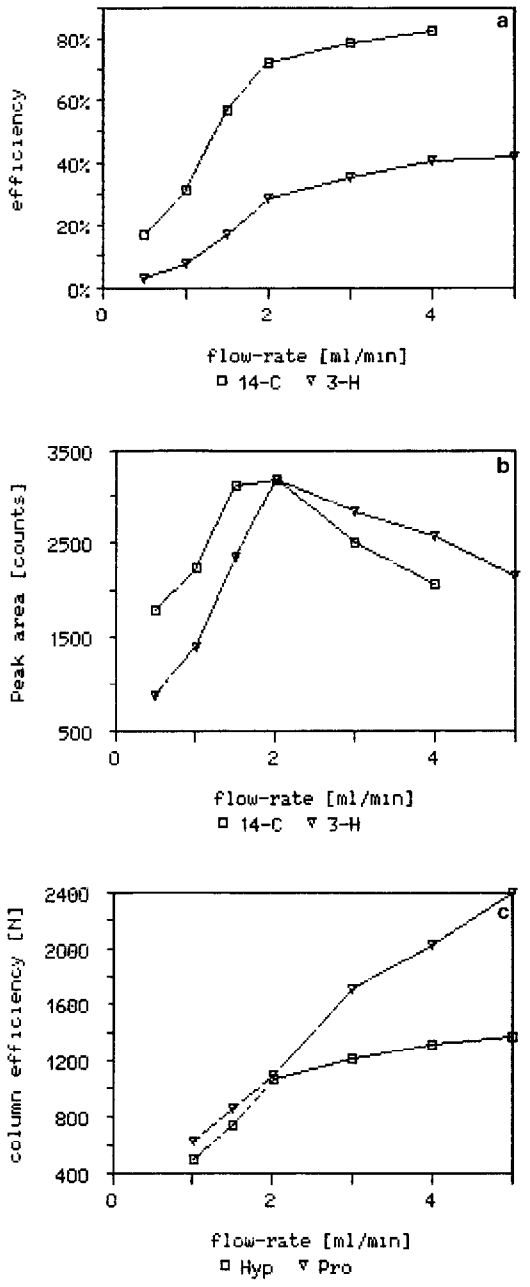

Fig 2 Relationship between the liquid scintillator flow-rate and (a) counting efficiency, (b) peak area and (c) the number of theoretical plates in the radiograms Radiograms of ^{14}C- or ^{3}H-labelled proline were recorded at a mobile phase flow-rate of 0 6 ml/min Flow-cell volume of radioactivity detector, 0.50 ml (Reproduced with permission from ref 20)

ing the radioactivity detector. The potential advantages of this set-up are at least two-fold: (i) water is excluded from the liquid scintillator phase and, therefore, extremely low mixing ratios may be used, even for mobile phases containing high salt concentrations, while maintaining satisfactory counting performance; (ii) band broadening is suppressed by the segmentation process

With this principle, the counting efficiency was found to be determined by, among other factors, the extraction efficiency of the radiolabelled analytes from the aqueous into the organic (scintillator) segments, which was explained from the relatively short range of the emitted β-particles in liquids compared with the dimensions of the liquid segments in the flow cells of the radioactivity detector. Accordingly, methods for enhancing the extractibility of the analytes, such as post-column ion suppression and ion-pair formation, should be used to take full advantage, as demonstrated in the determination of basic (amine-type) pharmaceuticals [19,24] and phenylhydantoin-derivatized amino acids [25]. In favourable cases, mixing ratios as low as 0.2 can be used at maximal sensitivity. This is demonstrated in Fig. 3, which shows the effect of extractability on the net peak area of [^{14}C]remoxipride (an amine-type pharmaceutical) as well as the perform-

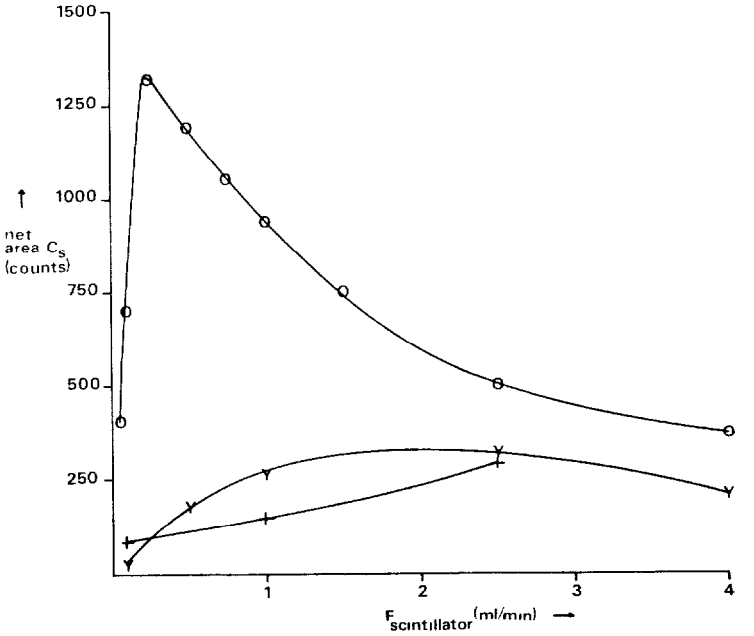

Fig 3 Peak area (^{14}C, in counts) as a function of the scintillator flow-rate Sample, [^{14}C]remoxipride (600 Bq); liquid scintillator, water-immiscible (+), water-immiscible with 1.0 m*M* sodium dodecylbenzenesulphonate (NaDBS) (o) and water-miscible (Y) Column, Cyano Spheri-5 (100 mm × 4 6 mm I D), mobile phase, acetonitrile–water (15.85, v/v), pH 2.2 with phosphoric acid at 1 0 ml/min Flow-cell volume of radioactivity detector, 63 μl NaDBS is used as ion-pair extraction agent to increase the extraction yield of the analyte from the aqueous into the organic scintillator segments *cf* (+) and (o) (Reproduced with permission from ref 23)

ance of a common water-miscible scintillator under otherwise identical conditions.

2.1.2.2. Flow-through heterogeneous scintillation counting. In flow-through heterogeneous (solid) SC, the flow cell in the radioactivity detector is packed with inorganic scintillator granules, such as yttrium silicate [20,26], europium-doped calcium fluoride [27,28] or cerium-doped lithium silicate [27, 29–32]. These materials are unaffected by a large range of solvents, although the combined use of calcium fluoride-packed cells with ammonia-containing eluents should be avoided since this salt is slightly soluble in these types of solvent [28]. Organic scintillators, such as anthracene and 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP), which were popular in the early days of heterogeneous counting, seems to have disappeared from this field of applications

The advantages of the heterogeneous counting mode over the liquid mode are generally recognized. In the former, the eluate can be recovered unmodified, while the volume of radioactive waste is at minimum. Furthermore, counting efficiencies of over 0.05 for ^{3}H have been reported recently, which is only three- to five-fold lower than for the liquid counting mode [33,34]. Table 2 summarizes the counting efficiencies for various solid scintillating materials, as reported by various workers. It should be stressed that mutual comparison of the various scintillator materials is hampered largely because of the differences in scintillator particle sizes and packing densities of the flow cells.

The most dominant disadvantage of flow-through heterogeneous counting,

TABLE 2

COUNTING EFFICIENCIES OF SOLID SCINTILLATORS

Type	Particle size		^{3}H	^{14}C	Ref
	mesh	μm			
Ce(Li)		90–125		0 44	29
		63–90		0 57	29
		38–63		0 62	29
		250–350		0 16	30
		250–350		0 40	88
	60–80		0.018–0.014	0 38 (S)[a]	31
	140–160		0 041–0.044	0.62	31
	160–180		0 069–0 062	0.71	31
	150			0.20	27
$EuCaF_2$	150–250			0 57 (S)	27
	45–100		0 035 (S)		89
	100–150		0 086 (S)		89
Y_2SiO_5		32–54	0 08	0 85	20

[a] S = static, *i e* at zero flow through the detector

however, is that the scintillator materials are not inert to certain types of analyte and are therefore prone to contamination. This was found, for instance, in the determination of ^{14}C-labelled sugars and corresponding esters in flow cells packed with lithium silicate granules [32] It was observed that the actual mean residence time of the analytes in the packed cell was in fact larger than the residence time calculated from the cell volume and the flow-rate alone. The difference was attributed to reversible adsorption of the analytes onto the silicate material. As a rule of thumb, it is normally advised not to use packed cells for analytes with molecular masses of over 600–1000. For these types of analyte, contamination may be explained from both particle filtering and the large number of reactive sites on polymeric molecules [35]. Accordingly, special care should be taken in protein-containing samples. Observations on the contamination of various types of analyte on solid scintillators have been collected in Table 3 together with possible remedies.

The optimal size for scintillator particles is *ca.* 40–60 μm, with respect to both the back-pressure generated across the packed cell and the counting efficiency. Smaller sizes result in excessive back-pressure, and these cells are more prone to contamination because of their larger specific area. In addition, photon scattering becomes dominant for densely packed cells, resulting in decreased light collection efficiency by the photomultiplier tubes and, hence, in a decrease in counting efficiency.

As a representative application, Fig. 4 shows the HPLC determination of ^{14}C-labelled nucleotides, with both UV and flow-through heterogeneous SC. The flow cells in the radioactivity detector (8 cm × 2 mm I.D.) was packed with CaF_2 (Eu) particles (*ca.* 150 mesh). By comparing the UV and ^{14}C traces, it can be seen that small bands can be obtained from the radioactivity detector. In more critical situations, a post-column make-up flow may be used to suppress additional band broadening in the flow cells of the radioactivity detector, rather than decreasing the cell volumes.

TABLE 3

ADSORPTION ON SOLID SCINTILLATORS

Scintillator	Sample	Remedy	Ref
Y_2SiO_5	Phospholipids	Flush with HNO_3 (20%)	90
CeLi	Sugars	Hot detergent and 0.1 *M* HCl flush	32
CeLi	Carboxylic acids	Repacking after 50 injections	88
CeLi	Phosphates, acetates	Repacking	29
$EuCaF_2$	Polymers	–	89
$EuCaF_2$	Glucuronides	Methanol, water or detergent flush	91
$EuCaF_2$	Ribonucleotides	Silanization	26
$EuCaF_2$	Nucleotides	–	28

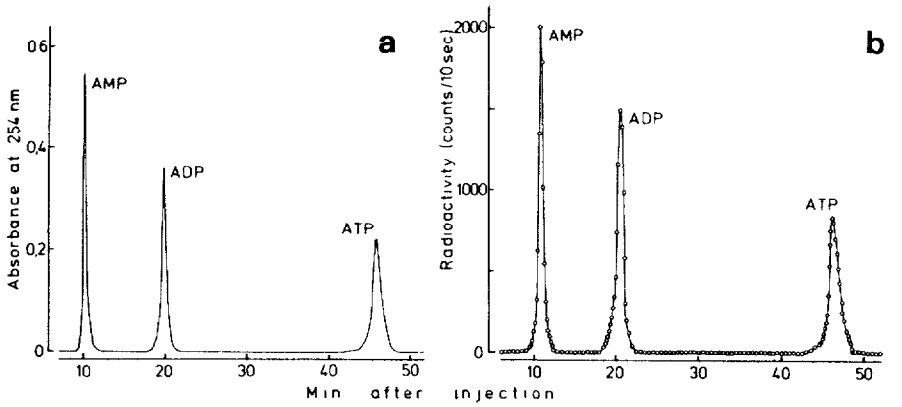

Fig 4 HPLC separation of [^{14}C]nucleotides AMP, ADP and ATP with (a) UV absorbance detection and (b) flow-through heterogeneous scintillation counting with CaF_2(Eu). Column, anion-exchange TSKgel DEAE-2SW (300 mm × 4 0 mm I D.), mobile phase, 50 m*M* potassium phosphate (pH 6 0)–acetonitrile (75 25, v/v) at 0 7 ml/min. (Reproduced with permission from ref 27)

An interesting alternative for flow-through heterogeneous SC was developed by Rucker *et al.* [35]. In this set-up, a detector cell was constructed by packing a cylindrical tube with axially aligned 0 1 mm I D cerium-doped lithium silicate fibres (Fig. 5). Owing to the hexagonal close packing geometry of the fibres, counting efficiencies for medium- and high-energy β-emitters were found to be satisfactory, *i.e.* values of 0.55 and 0.93 were found for ^{14}C and ^{32}P, respectively. A substantial reduction in back-pressure and contamination with polar analytes was observed for the fibre cell compared with the packed cell. For instance, at a methanol flow-rate of 2 ml/min, the back-pressure across the fibre cell and an yttrium silicate-packed cell (nominal particle diameter 25 μm) was 2 and 125 p.s.i., respectively. This system, therefore, has great potential for the detection of medium- and high-energy β-particles. Unfortunately, the ^{3}H counting efficiency

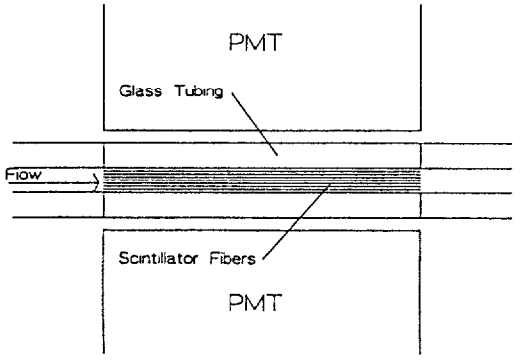

Fig 5. Glass scintillator-fibre flow cell, fibres are 100 μm I D (Reproduced with permission from ref 35)

was only 0.001, and no radiograms demonstrating the flow characteristics of the fibre cell were given.

2.2. *High-energy β-emitting radioisotopes*

Owing to the large (2 mm) mean range of the ^{32}P β-particles in condensed phases, this radioisotope can be detected with satisfactory efficiency using heterogeneous or even external SC. For the latter, a PTFE capillary is embedded in plastic scintillating material. Depending on the I.D. of the PTFE capillary, counting efficiencies of 0.80 [37] to 0.99 [38] may be obtained. ^{32}P can also be determined via the Cerenkov radiation produced upon the interaction of high-energy β-particles in solvents of high dielectric permittivity. The maximum emission intensity of this type of radiation is in the UV region, which does not normally match the optimal sensitivity range of common scintillation photomultiplier tubes. Mathews *et al.* [39] reported a 45% lower efficiency of Cerenkov counting compared with liquid SC.

^{32}P-labelled ATP is used extensively in the postlabelling of RNA and DNA adducts, as originally developed by Randerath *et al.* [40,41]. In most of these studies, separation of the ^{32}-P-labelled RNA and DNA constituents is performed by thin-layer chromatography (TLC) and radioactivity detection by autoradiography, but some radio-HPLC procedures have been reported recently [42,43]. In addition, ^{32}P-labelled phosphates are used as precursors in incubation studies. For example, [^{32}P]orthophosphate can be used to study inorganic pyrophosphate in cell culture medium [38], inositol phosphates [39] and leaf inositol phospholipids [44].

Fig. 6 shows the reversed-phase HPLC separation of a synthetic mixture of

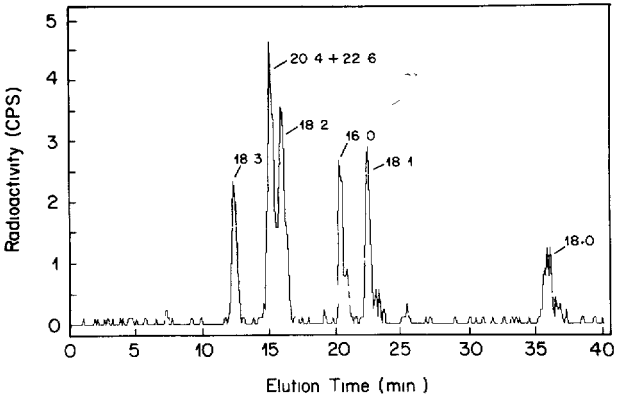

Fig. 6. HPLC elution profile of authentic molecular species of dimethyl ester derivatives of 1-acyl-lyso[^{32}P]phosphatidic acid Column, two ODS-2 columns in series (100 mm × 4 6 mm I D), mobile phase, methanol–water (88 12, v/v) at 0.8 ml/min, flow-through external plastic scintillation counting in a 250-μl flow cell (Reproduced with permission from ref. 37)

dimethyl esters of lysophosphatidic acids. Radioactivity detection was performed by flow-through external plastic SC in a 250-μl flow cell, at a counting efficiency of 0.80. This method facilitates the determination of the principal mechanism of formation of lysophosphatidic acids.

2.3. γ-Emitting radioisotopes

Compared with β-radioactivity, the detection of γ-emitting radioisotopes in HPLC column eluates presents few difficulties. This difference may be ascribed to the high penetration power of electromagnetic (γ) radiation in liquid or solid matter as opposed to particle (β) emission. As a consequence, matrix effects on the counting efficiency are almost absent in γ-counting, and good counting performance may be obtained by external counting techniques. The use of Na(Tl) scintillation outweighs by far the use of other methods, such as semi-conductivity detection [45], liquid SC of the low-energy radioisotopes ^{125}I and ^{99m}Tc [46] and plastic SC of 511-keV positron emitters ^{11}C, ^{13}N or ^{18}F [47,48]. For characteristics and applications of flow-through NaI(Tl) counting, the reader is referred to ref. 4. Evidently, these devices can be constructed at moderate costs from readily available components.

HPLC, in combination with radioactivity detection, is now extensively used, in particular for the identification of ^{99m}Tc-labelled radiopharmaceuticals, such as the group of bone-scanning [^{99m}Tc]diphosphonates [49–51]. [^{99m}Tc]Phosphonate complexes have been studied using anion chromatography and gel-permeation chromatography, which yields information on the charge and size of the complexes.

The combination of HPLC and flow-through γ-counting is one of the main methods for fast, efficient purification and metabolism studies of carrier-free radio-pharmaceuticals labelled with the short-lived positron emitters ^{11}C ($T_{1/2}$ = 20.4 min), ^{13}N ($T_{1/2}$ = 9.96 min), ^{15}O ($T_{1/2}$ = 2.03 min) and ^{18}F ($T_{1/2}$ = 109.7 min) [4,52]. These radioisotopes are detected via the 511-keV annihilation radiation. These labelled radiopharmaceuticals are extensively used as biochemical tracers for non-invasive study of the distribution in living organisms, using external scanning devices (positron emission tomography).

Fast HPLC analysis of metabolic products was mandatory for the *in vivo* determination of the short-term metabolic fate of [^{13}N]ammonia in rat liver, as studied by Cooper *et al.* [53]. An example is shown in Fig. 7, which gives the elution profile of ^{13}N-labelled metabolites obtained from deproteinized liver, only 15 s after a bolus injection of [^{13}N]ammonia into the portal vein of an anaesthetized adult male rat. Note that the analysis time is *ca.* 24 min, which is *ca.* 2.5 times as long as the half-life of ^{13}N. Accordingly, ^{13}N peak areas are corrected for radioactive decay during analysis. The authors state that for studying short-term nitrogen metabolism. ^{13}N should be preferred to the stable isotope ^{15}N. This was argued from the unphysiologically high ^{15}N concentrations needed

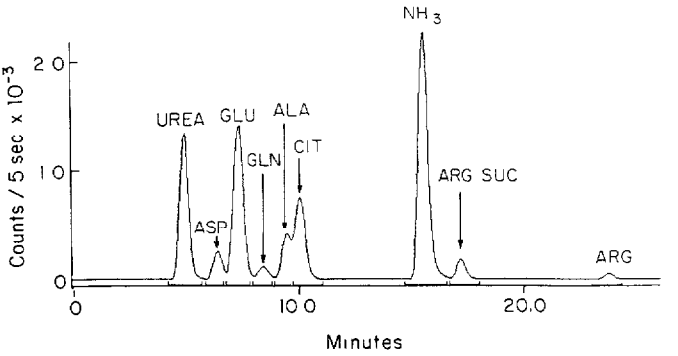

Fig 7 HPLC elution profile of ^{13}N-labelled metabolites obtained from deproteinized rat liver 15 s after a bolus injection of trace amounts of [^{13}N]ammonia into the portal vein of an anaesthetized adult male rat Column, Partisil 10 SCX (250 mm × 4 6 mm I D); mobile phase, 5–20 m*M* potassium phosphate hydrochloric acid (gradient pH 2 55 to 3 5), detection by flow-through γ-counting (Reproduced with permission from ref 53)

in biological experiments to obtain a measurable enrichment in the metabolic products. By contrast, ^{13}N can be used at extremely low concentrations since it does not occur under natural circumstances. Needless to say this type of work should be performed close to a ^{13}N-production cyclotron facility.

The mutual benefits of HPLC and radioactivity counting techniques can further be demonstrated by the HPLC determination of cisplatin and its metabolic products. Although several HPLC methods for the determination of this potential anti-tumour agent in biological samples have been reported, most of the detection methods applied lack sensitivity as well as selectivity. A possible solution to this problem was given by Mauldin *et al.* [54], who used ^{3}H-labelled ligands. In the method described by Baldew *et al.* [55], cisplatin was synthesized using ^{195m}Pt ($T_{1/2}$ = 96 h) to a specific activity of 15 MBq/mg cisplatin. Flow-through radioactivity detection was by liquid SC, although external SC might also have been applicable since this radioisotope is also a γ-emitter. The labelled compound was administered to male wistar rats. Blood and urine samples were collected at selected time intervals. An example is given in Fig. 8. In this study, a 1.0-ml cell volume was used at an scintillator/eluate mixing ratio of 5 (total flow-rate 1.55 ml/min), at a counting efficiency of 0.61. Under these conditions, a detection limit of 150 Bq was claimed, corresponding to 10 ng of cisplatin per ml of plasma. This compares favourably with alternative detection methods, such as electrochemical detection, quenched phosphorescence detection or ICP atomic emission spectrometry. More importantly, the radiolabel unequivocally identifies the cisplatin metabolites in the radiogram.

3 RADIO CAPILLARY ZONE ELECTROPHORESIS

With the rapidly growing interest in the separation of ionizable biomolecules

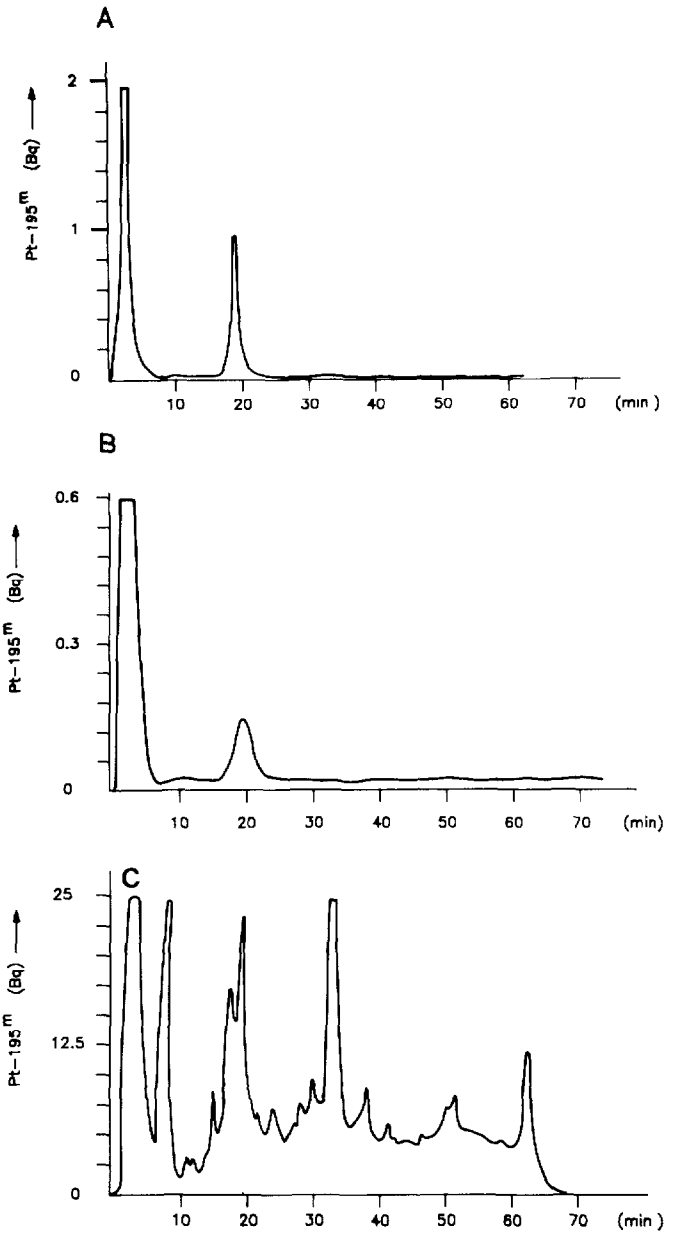

Fig 8. HPLC radiograms of cisplatin metabolites in rat plasma ultrafiltrate (A and B) and a urine sample (C) obtained after intravenous treatment with [^{195m}Pt]cisplatin. Column, ODS (100 mm × 3 0 mm I D), mobile phase, gradient elution based on 10–60 m*M* sodium phosphate buffer (pH 2 6), 5 m*M* sodium dodecylsulphate and 2-propanol (0–25%, v/v) at 0 25 ml/min; detection by flow-through liquid scintillation counting The first eluting peak corresponds to the parent compound, cisplatin (Reproduced with permission from ref 55.)

by capillary zone electrophoresis (CZE), the construction of flow-through radioactivity detectors for these types of separation has received some attention [56, 57]. These detectors differ from flow-through detectors employed in radio-HPLC. First, in order to preserve the chromatographic integrity, extremely small cell volumes of less than 200 nl are used. Second, as noted by Pentoney *et al.* [57] in CZE the separated sample zones travel with different speeds through the detector cell according to their individual electrophoretic mobilities. As a consequence, the mean residence time in the detector cell must be determined for each individual component for accurate calibration. Finally, to improve the resolution of neighbouring sample zones, so-called spacing constituents can be used. To some extent, this latter aspect is related to the post-column segmentation techniques used in radio-HPLC, based on solvent, air or gel segmentation mentioned previously.

Fig. 9 shows a detailed view of a flow cell constructed by Kaniansky *et al.* [56]. The sensing part of the cell is made of a plastic scintillator with a 0.3 mm I.D. capillary channel drilled through, which equals the I.D. of connecting tubing to prevent back-mixing of the eluate in the cell Cell volumes of 70 or 200 nl can be selected by using either 1 or 3 mm thick scintillating layer, respectively. A counting efficiency of 0.13–0.15 was found for ^{14}C. In principle, this value may be improved by reducing the I.D. of the cell. The beneficial role of spacing constituents on the resolution is shown in Fig. 10. In this example, ^{14}C-labelled acetate and glutamate were separated with 2-bromopropionate as spacing constituent.

Two relatively simple-to-construct flow cells are described by Pentoney *et al.* [57]. In their first opinion, a 2-mm section of a 100 cm × 100 μm I.D. fused-silica capillary tube was positioned close to a commercially available CdTe semicon-

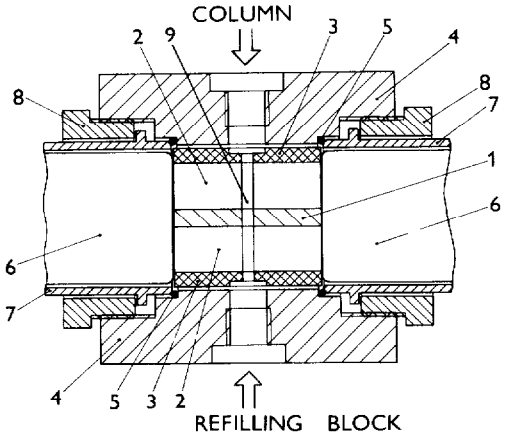

Fig 9 Design of flow cell in radioactivity detector for capillary isotachophoresis 1 = Layer of plastic scintillator, 2 = transparant plastic material compatible with the plastic scintillator, 3 = black layer of plastic material; 4 = duralumin assembly, 5 = O-rings for light-tight connection of the photomultiplier tubes (6) to the detection cell, 7 = metal housings for the photomultiplier tubes, 8 = fixing screws, 9 = capillary channel identical in diameter with the I D of the capillary tube used in the column (Reproduced with permission from ref 56.)

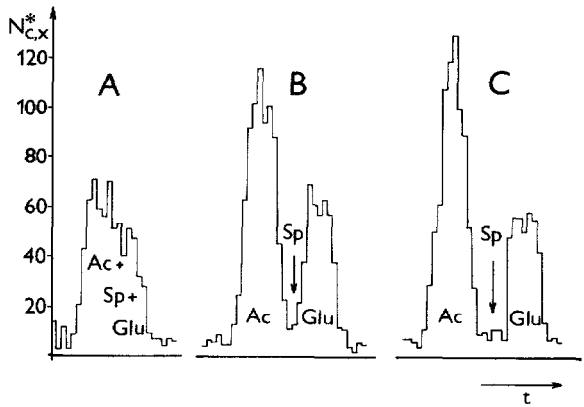

Fig. 10 Capillary isotachopherograms with flow-through ^{14}C detection with the cell shown in Fig 9, showing the beneficial rol of the spacing constituent 2-bromopropionate (Sp) in resolving neighbouring zones of ^{14}C-labelled acetate (Ac) and glutamate (Glu) The amounts of the spacer are (A) 1 nmol, (B) 1 5 nmol and (C) 2 nmol, giving 2, 3 and 4 mm zone lengths, respectively (Reproduced with permission from ref 56)

ductor detector In the second option, the fused-silica tubing was passed through a hole drilled in a plastic scintillator. The sensing part of both detectors amounts to 15 nl. Both systems were tested with ^{32}P-labelled compounds. The ^{32}P counting efficiency was 0.26 and 0.65 for the semiconductor and plastic scintillator detector, respectively. Efficiencies are somewhat lower than with liquid SC because some of the ^{32}P β-particles are stopped in the fused-silica material and do not reach the scintillator (the average range of the ^{32}P β-particles in silica amounts to 0.95 mm). For this reason, the efficiencies for ^{14}C, ^{35}S and, in particular, ^{3}H are expected to be substantially lower owing their lower β-energies.

In Fig. 11, some electropherograms of ^{32}P-labelled triphosphate derivatives are given, recorded with the plastic scintillation detector. In this example, flow-programming was used to increase the sensitivity of flow-through counting in the region where the analytes of interest eluted. To this end, the voltage potential across the separation capillary was reduced from -20 to -2 kV upon elution of the labelled compounds through the couting cell. Note the corresponding increase in counts recorded on the vertical axis of both figures.

4 RADIO GAS CHROMATOGRAPHY

Historically, GC in combination with radioactivity detection has never reached the popularity of, say, radio-HPLC. Apart from studying the radiochemical purity of radiolabelled material, the few applications of radio-GC reported in the literature are almost exclusively concerned with the determination of ^{3}H- or ^{14}C-labelled analytes. Relevant applications include the determination of radiolabelled lipids [58], steroids [59–61], organic acids and carbohydrates [62,63], choline and acetylcholine [64] and pharmaceuticals [65].

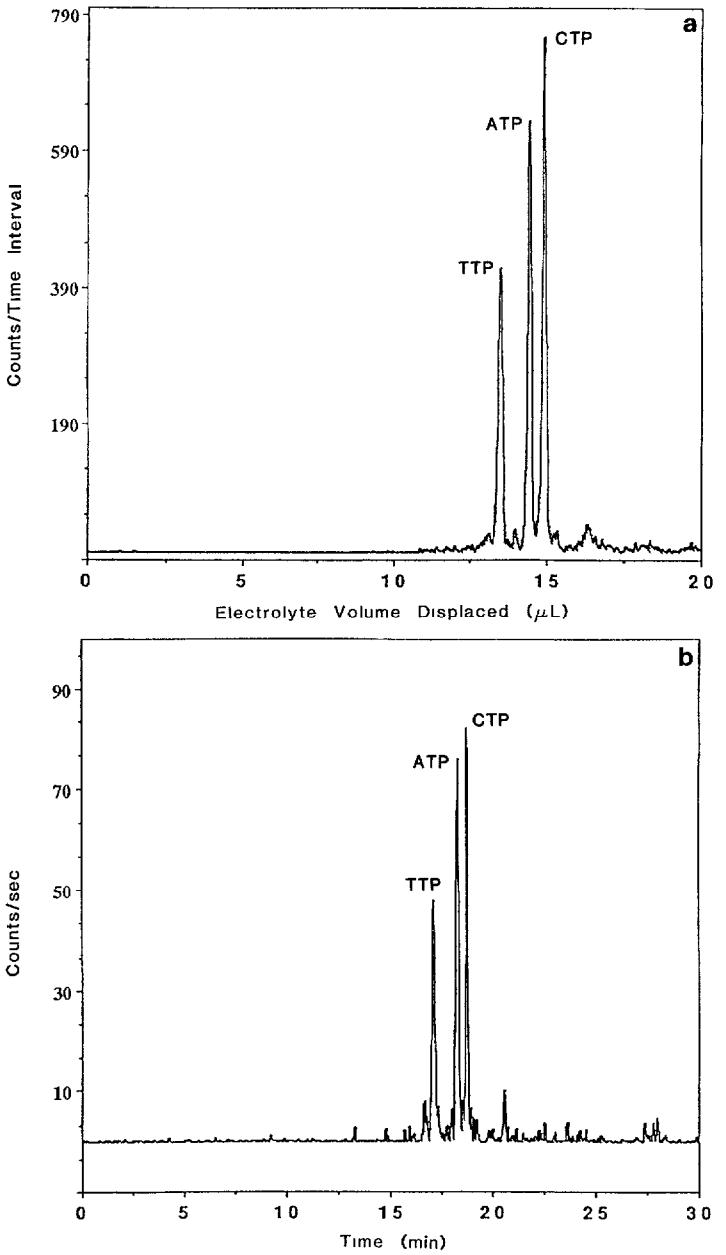

Fig 11 (a) Flow-programmed capillary zone electropherogram of α-^{32}P-labelled triphosphates of thymidine (TTP), cytidine (CTP) and adenosine (ATP) obtained by injecting *ca* 700 Bq (2×10^{-8} *M*) of each component onto the capillary The separation was flow-programmed by applying a constant potential of -20 kV until the radiolabelled sample approached the detection volume and then reducing the potential to -2 kV as the sample zone traversed the detection region. Note that the detector signal has been plotted as a function of electrolyte volume displaced, resulting in a time-compressed abscissa over the flow-programmed region of the electropherogram The operating current was 38 μA at -20 kV and 3.8 μA at -2 kV. Capillary, fused silica, 100 μm I D , supporting electrolyte, borate buffer (pH 8.1, 0 20 *M*). (b) Capillary electropherogram as in (a), except that elution was at constant voltage applied (-20 kV). (Reproduced with permission from ref 57)

The usage of radio-GC in pharmaceutical and biomedical studies is not likely to increase, largely because selective, non-radioactive methods are readily available; the coupling of high-resolution capillary GC with selective detectors, such as mass spectrometers or Fourier transform infrared spectrometers, is now very common. By contrast, radioactivity counting of ^{3}H and ^{14}C in GC effluent streams is inherently more complicated and laborious, in particular for the discontinuous (off-line) sampling techniques. This is probably best illustrated by the many various options developed for discontinuous sampling of GC effluents [2]. Some of them are still in use and may be adapted by other workers at low cost, especially in situations when sample throughput is low and optimal chromatographic resolution is of less concern [64].

Combustion of the GC effluent prior to counting is required in most radio-GC techniques. Accordingly, the most important parameter to be considered, irrespective of the counting mode, is the combustion efficiency for rapid conversion of the analytes into CO_2 (for ^{14}C) and H_2O or H_2 (for ^{3}H). Partial combustion normally results in poor peak shapes and increased background count rates, owing to column bleeding of radioactivity. Unfortunately, few systematic studies on this matter are available in the open literature. Traditionally, combustion techniques have been based on the use of CuO combustion tubes, heated to *ca.* 800°C Rodriguez *et al* [67] studied the performance of combustion tubes (60 cm × 4 mm I.D.) packed with quartz granules (0.5–1 mm diameter) with CuO particles (0.1–0.3 mm diameter) or CuO wire (0.4–0.5 mm I.D.). In both cases, the back-pressure across the tube rapidly increased after three injections of ^{14}C-labelled dibromomethane. This was attributed to surface exhaustion of the CuO beds, leading to a decrease in permeability and an increase in the adsorptive capacity of the packing. As a result, the split ratio of the GC effluent to the radioactivity detector and the flame ionization detector changed from 57/43 to less than 50/50, whereas the recovery of radioactivity from the combustion tube declined, as indicated by the poor peak shapes and the increased background count rate between radioactive peaks. It was further shown that these problems could to a large extent be avoided by optimizing the CuO/quartz ratio and by the addition of a small amount of oxygen to the carrier gas.

Baba *et al.* [68] determined the combustion efficiency of ^{14}C-labelled hexadecane, testosterone and histidine (the latter after derivatization with pentafluoropropionic anhydride) as a function of the amount of CuO, the carrier gas flow-rate and the amount of analyte. The combustion assembly consisted of a 180 mm × 5 mm I.D. quartz tube, packed with CuO wire (5 mm × 1 mm I.D., total weight 2–10 g). The tube was positioned in an electric oven furnace at 800°C. It was concluded from the experiments that, even for the less combustable testosterone and histidine derivatives, this set-up could quantitatively oxidize the analytes (up to 200 μl hexadecane), virtually unaffected by the carrier gas flow-rate It was observed, however, that recoveries of testosterone were generally less than 90%, which could be attributed to adsorption of the compound on the injection port and the stainless-steel capillaries.

More recently, it was shown that combustion using the flame ionization detector of the gas chromatograph is also feasible [63,66,69,70], although in this case large volumes of water should be trapped and the counting device should be capable of accommodating high flow-rates [66].

Whatever the combustion method, heating of connecting lines appears to be critical in preventing adsorption of the analytes on the inner walls and radioactivity bleeding.

The combustion techniques simplify dual-isotope detection of ^{14}C and ^{3}H because the radioisotopes are chemically separated. The inherent drawback of combustion is that it is restricted to these two radioisotopes. To the author's knowledge, no applications of flow-through ^{32}P or ^{35}S counting in GC have been published.

Following combustion, the radioactivity can be recorded by liquid or heterogeneous SC or by gas-proportional counting, which is now certainly the most popular counting technique. For gas-proportional counting of ^{3}H, water is first converted into H_2 by reduction over hot iron. The gaseous stream is then led through a tube packed with magnesium perchlorate, silica gel or calcium chloride to remove traces of water. Before it enters the counting tube, a counting gas is mixed with the stream, which normally consists of propane, methane or methane in argon (P10 gas). The principal advantage of gas-proportional counting over SC (see below) resides in the almost optimal counting efficiencies (>0.9 for ^{14}C, >0.6 for ^{3}H) in combination with low background count rates (generally less than 4 cpm). Its main disadvantage is the short counting time, which is set by the total volume of the counting tube and the total flow-arte of the combined gases.

If radio-GC is used only infrequently, alternative methods such as based on SC can be employed. To this end, radioactive carbon dioxide in the column effluent may be collected in a suitable solvent (*e.g.* amines [63,70]) or an alkaline liquid scintillator [66] for subsequent off-line SC. These systems also work well for trapping tritiated water. As with gas-proportional counting, it is important to heat all transfer lines in order to prevent condensation of the gases before they enter the trapping solvent. Alternatively, the analytes can be trapped without combustion on cartridges packed with suitable adsorbers [64] or on TLC plates [59].

5 RADIO PLANAR CHROMATOGRAPHY AND GEL ELECTROPHORESIS

5.1. General

Planar techniques (*i.e.* TLC, paper chromatography and gel electrophoresis) continue to play an important role for the separation of radiolabelled compounds. Except for gel electrophoresis, these techniques are often used as a sample pretreatment step for HPLC separation.

The widespread popularity of radio planar chromatography stems from the

large batches of samples that can be treated in a single run, the fact that normally all radioactivity can be visualized irrespective of the form of the radiolabelled material, *i.e.* whether particulate, colloidal or dissolved and, finally, the relative ease and low costs involved in qualitative determination of the spatial radioactivity distribution.

Admittedly, there are also some general drawbacks to radio planar chromatography. Chromatographic resolution is normally inferior to that of the techniques mentioned in previous sections; apart from obvious chromatographic parameters, such as the type of chromatographic support and mobile phase composition, resolution in radio planar chromatography is determined by the range of the β-particles emitted from the support. In general, resolution decreases at increasing range (*i.e.* energy) of the β-particles. As a consequence, resolution is at optimum for the low-energy β-emitter ^{3}H, although at the cost of counting efficiency [2]. Resolution deteriorates for the higher-energy emitters, such as ^{32}P, but counting efficiencies are satisfactory. Close contact between the plate and detection medium is thus a prerequisite to improve resolution and counting efficiency. Mohamed *et al.* [71] employed polyethylene glycol solution for shrinking polyacrylamide gels to one quarter of their original area. In this way, sharp and concentrated bands were obtained that gave improved visualization by autoradiographic detection.

Most techniques for radioactivity detection in planar chromatography are regarded as qualitative methods of investigation. Unfortunately, very few systematic studies are available in the open literature on reproducibility, linearity and detection limits of the various detection principles.

To obviate the drawback of getting qualitative results only, zones of interest from the chromatographic adsorbent may be selected for subsequent liquid SC if pure β-emitters are involved. Indeed, this so-called plate sectioning or plate scraping principle is still widely used when quantitative data have to be obtained. The adsorbent, however, may adversely affect the counting results [2,72,73]. Reimschussel and Kubik [72,73] investigated the adsorption behaviour of ^{14}C-labelled compounds and scintillator fluorophores on silica gel granules varying in grain size, pore diameter and specific surface area. One of the general observations in this study was that the larger the grain size and the narrower the pores, the lower the counting efficiency. As an alternative for direct liquid SC, the analytes can be eluted from the adsorbent or combusted into CO_2 (for ^{14}C-labelled analytes) and H_2O (for ^{3}H-labelled analytes) prior to counting.

In addition to destructive SC, various non-destructive methods are in common use for the determination of spatial radioactivity distribution: spark chamber, scanning detectors, autoradiography and position-sensitive analysing systems [2,6,8,74]. Spark chambers and scanning detectors have limited applicability, and only autoradiography and position-sensitive analysers will be discussed here.

5.2. *Autoradiography*

5.2.1. *General aspects of autoradiography*

Autoradiographs are obtained by placing the dried chromatogram or gel in direct contact with a photo-sensitive film for a suitable period of time. A latent image in the film emulsion is thus obtained, which can be visualized by film development and densitometry. Proper handling, film exposure and film processing are required to achieve an adequate response (for reviews on basic principles of autoradiography see refs. 1, 2 and 75).

It has been shown that the sensitivity of the film can be improved considerably by chemo-physical treatment of the film, *i.e.* short exposure of the film to high-intensity light (preflashing; ref. 75), by baking the film in hydrogen–nitrogen gas before exposure (hypersensitation; ref. 76) and by low-temperature exposure, generally at $-80°C$.

Three methods for film exposure can be distinguished (see also Fig. 12): direct exposure, fluorography and intensifying screens. Selection of a specific method will be determined by the type or radioisotope and the amount of radioactivity to be detected. Fig. 13 given an overview of some criteria for the selection of an appropriate method.

5.2.2. *Direct autoradiography*

Direct autoradiography (see Fig. 12) is applicable for the detection of medium- to high-energy radioisotopes. For the low-energy emitter ^3H, about half of the

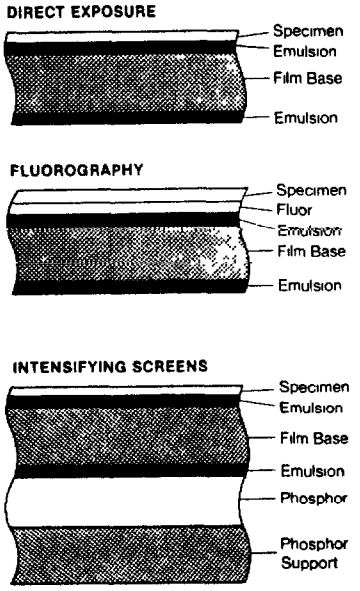

Fig 12 Arrangements for autoradiographic exposure methods (Reproduced with permission from ref 75)

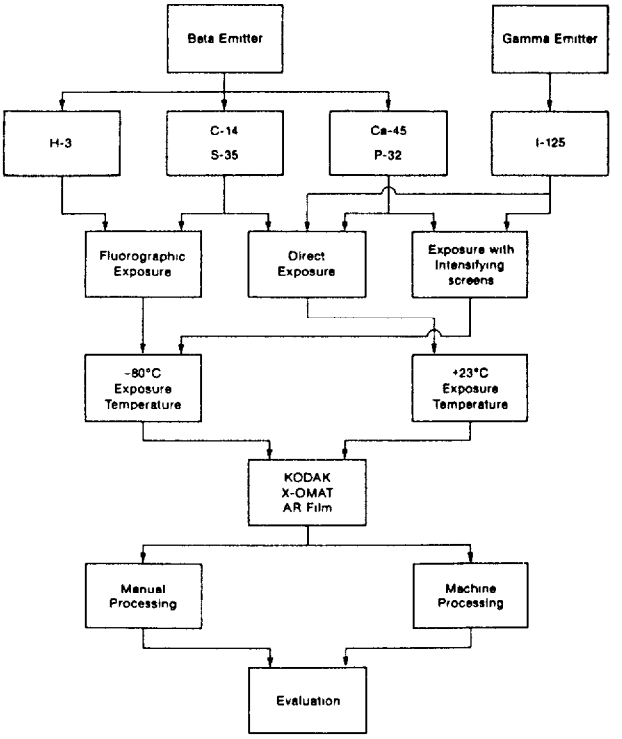

Fig 13 Flow diagram for selection of autoradiographic exposure methods. (Reproduced with permission from ref 75)

emitted β-particles are stopped within 0.8 μm of silica on the thin-layer plate. The thickness of silica layers of most commercially thin-layer plates is typically 200 μm, which explains the relative intensitivity of direct autoradiography for ^{3}H detection. In this context, it is felt that the overall performance of direct autoradiography may benefit from the narrower adsorbent layer thickness distribution in high-performance TLC plates compared with conventional TLC plates, but no systematic studies on such comparison have been reported. A relative standard deviation of over 7% in net peak areas has been reported under ideal conditions, *i.e.* by employing very thin radiolabelled foils under carefully standardized conditions for film exposure and film development [77].

5.2.3. Fluorography and intensifying screens

Fluorography involves embedding the chromatogram with a suitable fluorophore [79–81]. The function of the flurophore is to convert the β-particle energy into light pulses, which in turn can expose the film. Fluorography is especially suitable for detection of low-energy emitters ^{3}H and ^{63}Ni since, owing to the short range of these β-particles in the chromatographic support, direct autoradiography for these radioisotopes is very inefficient. Roberts [79] reported mini-

mal levels of ^{35}S or ^{14}C that could be detected by direct autoradiography and fluorography: 400 and 100 dpm/cm^2, respectively, for 24-h exposure (four-fold decrease). In contrast, for ^{3}H these levels were 20 000 and 500 dpm/cm^2, respectively, corresponding to a forty-fold decrease.

There seems to be agreement that 2,5-diphenyloxazole (PPO) is the most suitable fluorophore, although sodium salicylate is sometimes applied because of its higher solubility in water and also for health risk reasons (salicylate is less toxic than PPO). Fig. 14 compares autoradiograms obtained from ^{3}H-labelled proteins after electrophoretic separation on a polyacrylamide gel and transfer to a thin low-density nitrocellulose membrane prior to exposure Employing direct autoradiography and fluorography with PPO or sodium salicylate it was found that the maximum enhancement of densitometric tracing relative to direct autoradiography was 100-fold or 200-fold for salicylate and PPO treatment, respectively.

Intensifying screens improve the detection of high-energy β-emitters, such as ^{45}Ca and ^{32}P, and low-energy γ-ray emitters, such as ^{125}I. The screen phosphor accepts the ionizing radiation that escapes through the first emulsion layer, film base and second emulsion layer, respectively, and converts it into light pulses. In this way, more efficient detection takes place allowing shorter exposure times

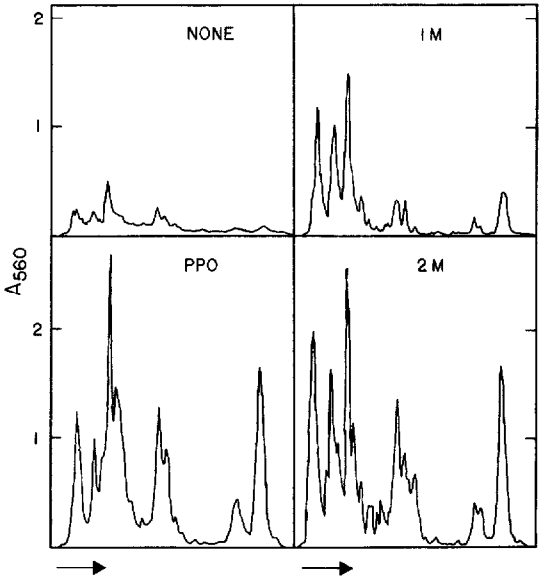

Fig. 14 Fluorography of ^{3}H-labelled proteins blotted on nitrocellulose membrane Aliquots containing 1 3 10^5 cpm of ^{3}H-labelled protiens were fractionated by gel electrophoresis in replicate lanes and blotted on nitrocellulose Replicate strips were treated with 1 or 2 *M* sodium salicylate solution, with 0 4% PPO in methylnaphthalene, or left untreated The strips were exposed to film for 4 h (PPO), 8 h (salicylate) or 48 h (no treatment). Absorbance due to each protein band represented in the film was determined by densitometric tracing The arrows indicate the direction of electrophoresis in the original gel (Reproduced with permission from ref 81)

5.3. Position-sensitive analysers

Position-sensitive analysers provide information on the radioactivity distribution on a chromatographic plate in just one measurement without mechanical scanning. They have been developed from the technology of large-scale computerized radiation detectors utilized in particle physics for detecting particle trajectories in space. It is expected that, in the near future, most of the mechanical scanning detectors will be replaced by position-sensitive analysers.

At present, there seems to be no consensus with respect to the general name of these systems; imaging scanners [82–84], linear analysers [8,74] and thin-layer scanners [6] all refer to position-sensitive analysers.

All instruments employ gas-proportional counters with either a resistive anode wire [84] or a position-sensitive counting wire in combination with a coiled delay line [74]. Fig. 15 give a schematic diagram of a resistive anode imaging scanner. Note that the scanner views the entire TLC lane simultaneously. A small flow of P10 counting gas, argon–methane (90:10) is continously purged from a cylinder through the detector. Ionization radiation interacts with the P10 gas to produce electron avalanches. The electrons are accelerated toward the high-voltage anode, and the collected charge is divided towards both ends of the resitive anode in proportion to the position of the incident electron avalanches near the anode. After amplification digitization and detection, this position is calculated by the microcomputer. The numbers of events are accumulated and stored in specific memory locations corresponding to that position.

In another design, instead of a resistive anode, a delay line is employed, adjacent to and parallel to a coiled delay line. As the electrons produced in the P10 gas are collected by the counting wire, a positive ion cloud remains and induces a

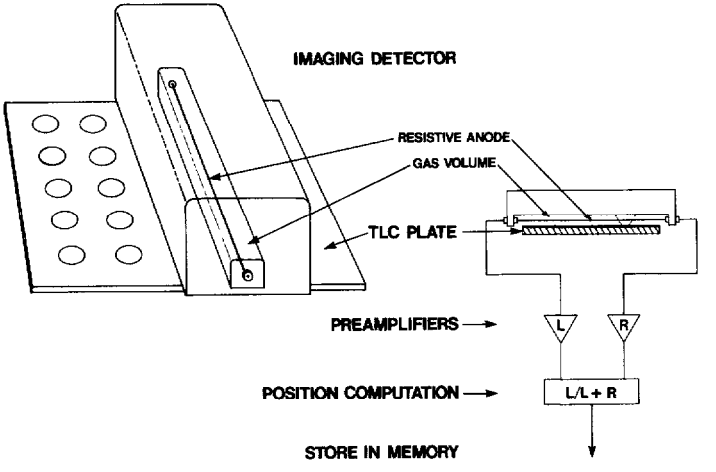

Fig 15 Schematic diagram of a resistive anode imaging gas-proportional counter (Reproduced with permission from ref. 84.)

signal on the delay line. This signal then propagates with a fixed speed in both directions along the delay line. The time difference between arrival times of the signal at both ends of the delay line is recorded and can be used to calculate the origin of the signal on the delay line. Using fast electronics, time differences from 10^{-9} to 10^{-6} s can easily be recorded, corresponding to positions from 0 25 to 250 mm on the counting wire [74].

Since these position-sensitive analysers operate in the proportional region, the electron avalanches are limited to locations defined by the position of the incident ionization radiation, *i.e.* directly above those at which particles or γ-rays are emitted from the chromatographic plate. In combination with fast electronics, this allows high resolution to be obtained, in particular for low-energy β-emitters. Typical resolutions are: 0.5 mm (^{3}H), 1 mm (^{14}C, ^{35}C, ^{33}P) and 3 mm (^{32}P, ^{125}I, ^{99m}Tc). Typical efficiencies vary from 0.5% to 2% (^{3}H), 20% (^{14}C, ^{35}S and ^{33}P), 5% (^{99m}Tc, ^{125}I) and *ca.* 40% (^{32}P, ^{99m}Tc, ^{131}I, ^{18}F) at background count rates of *ca.* 50 cpm per 250 mm [82].

Rexa *et al.* [78] found a certain non-homogeneity in the ^{14}C counting efficiency at the ends of a 20-cm-long position-sensitive analyser in the longitudinal direction At a distance of 1 cm from the ends, the counting efficiency dropped by *ca.* 13% with respect to the maximum in the detector centre.

6 ACCELERATOR MASS SPECTROMETRY

Radioisotopes with long half-lives can be detected at far higher efficiencies by atom counting (*i.e.* by mass spectrometry [85]) than by decay counting techniques, such as scintillation and gas-proportional counting. During the past decade, accelerator-based mass spectrometric techniques have been developed for the atom-counting of the long-lived radioisotopes ^{10}Be, ^{14}C, ^{26}Al and ^{36}Cl at their natural abundances. For a very interesting outline of the principles, performance and applications of this relatively new technique, ref. 86 is recommended reading. At present, ^{14}C atom counting can be performed routinely by accelerator mass spectrometry (AMS). The potential of AMS in combination with GC separation for the determination of ^{14}C at natural abundances in biological compounds is discussed by Gillespie [87]. The author anticipates that this technique will be usable for the radiocarbon dating, after derivatization, of amino acids or fatty acids.

7. CONCLUSIONS

Traditionally, radiochromatographic methods have the reputation of being very laborious and time-consuming, both in data acquisition and data handling. In addition, the presupposed hazards of radioactivity, the scarcity of radiolabelled compounds and the costs involved in the disposal of radioactive waste may prevent the use of radiolabelled material in solving an analytical problem. It can

further be argued that during the past two decades or so, the combination of modern high-efficiency separation methods with sensitive and selective detection principles (*e.g.* stable isotope mass spectrometry, Fourier transform infrared spectrometry, fluorescence detection based on selective derivatization) has reduced the need for ultimate selectivity in detection.

It is therefore surprising to find that, at present, radioisotopes are still extensively used in virtually all areas of chromatography, with the possible exception of GC

One explanation for this resides in the vast amount of effort that manufactures have spent in the adaptation of radioactivity detectors to modern chromatographic techniques – flow-through scintillation counters and position-sensitive analysers are the most important examples in this field – and the introduction of data acquisition and data handling systems based on readily available computer systems, resulting in a wide choice of easy-to-use radioactivity detectors with satisfactory and flexible performance at moderate prices. Owing to strong competition between manufacturers, prices of radioactivity detectors still seem to be decreasing. Consequently, radiochromatography is losing much of its negative image.

Along with the instrumental improvements, the potential of using of radiolabelled compounds is becoming more and more recognized. The general applicability of these compounds (general in terms of type of analyte and sample matrices) is unsurpassed because radioactivity detection is essentially an elemental analysis. In contrast, with alternative detection principles much time is normally spend in the development and validation of a new, single application.

8 SUMMARY

Recent advances in the chromatographic analysis of radiolabelled compounds are discussed, with emphasis on the optimization of radioactivity detection in modern chromatographic techniques. Applications are mentioned only infrequently. The state-of-the-art of common radioactivity detection methods is reviewed, as well as promising new detection principles. The latter include flow-through liquid scintillation counting of reversed-phase column eluates based on post-column extraction and solvent segmentation, flow-through heterogeneous counting using a flow cell constructed from axially aligned scintillation fibres, flow cells especially constructed for the detection of β-emitting radioisotopes in capillary zone electrophoresis, position-sensitive analysers employed in radio planar chromatography and, finally ^{14}C atom counting (as opposed to ^{14}C decay counting) by accelerator mass spectrometry.

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