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MODERN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–RADIOIMMUNOASSAY STRATEGIES FOR THE STUDY OF EICOSANOIDS IN BIOLOGICAL SAMPLES

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LIST OF ABBREVIATIONS

$C_{20:4\omega6}$	Arachidonic acid
ED	Electrochemical detection
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy

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GC Gas chromatography
HETEs Hydroxyeicosatetraenoic acids
HPLC High-performance liquid chromatography
HRP Horseradish peroxidase
LTs Leukotrienes
MS Mass spectrometry
PGI_2 Prostacyclin
PGs Prostaglandins
PH Portal hypertension
RIA Radioimmunoassay
TXB_2 Thromboxane B_2
UV Ultraviolet

1 INTRODUCTION

As is well known, in many instances the combination of suitable analytical techniques (directly or through the appropriate interfaces) enhances their individual attributes, providing an array of useful so-called hyphenated techniques such as gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC)–MS, HPLC–ultraviolet (UV) detection, GC–Fourier transform infrared spectroscopy (FTIR), etc., which are more powerful in terms of overall assay accuracy and precision than their individual components. These techniques allow the biomedical researcher to venture into ever more demanding fields of application, where complex biological sample matrices often pose seemingly insurmountable problems

The combined HPLC–radioimmunoassay (RIA) procedures, whereby the HPLC provides highly purified fractions for off-line RIA, efficiently couple together the best of both systems while contributing to minimizing some of the disadvantages regarding insufficient detection sensitivity in HPLC and lack of specificity in RIA. Thus, HPLC greatly simplifies the separation of complex mixtures in biological extracts whereas RIA can detect and quantify at very high sensitivity the HPLC-separated components The application of HPLC–RIA procedures to different types of compounds has been briefly summarized for the period 1975–1984 [1,2]. This review will mainly emphasize the work carried out in this field within the last four years and will be concentrated on the determination of metabolites of archidonic acid, collectively known as eicosanoids on account of their structural backbone of twenty carbon atoms The reasons behind the selection of this family of physiologically relevant compounds amenable to HPLC–RIA determinations lie in the extended experience of the authors in this area and the fact that in their analytical complexity they are representative of the type of problems one may encounter in HPLC–RIA procedures with other families of compounds in biological samples such as

peptides, steroids and pharmaceuticals The most important factors underlying the significance of eicosanoids as model compounds for the discussion of combined HPLC–RIA strategies can be summarized in (a) their wide range of different types of metabolites of biological interest, (b) the need for the separation of chemically similar substances whose actions can be functionally different and even sometimes antagonistic, (c) the various options for quantification depending on the sensitivity and/or minimization of analytical artifacts and (d) the determination in biological samples of metabolites of different chemical characteristics with the usual associated problems of interferences, need for purification, etc

Eicosanoids arise from the arachidonic acid ($C_{20\ 4\omega6}$) released from cell membrane phospholipids, which is metabolized by the cyclo-oxygenase and lipoxygenase enzyme systems to a very interesting and diverse array of compounds These can be subdivided into the prostaglandins (PGs) and prostaglandin-like compounds, the hydroxyeicosatetraenoic acids (HETEs) and the leukotrienes (LTs), as summarized in Fig 1

The HPLC purification and separation of eicosanoids, on both reversed- and normal-phase columns, especially the former, has become commonplace with a substantial number of publications appearing regularly in the specialized literature, and there are also reference HPLC methods available for general separation of prostaglandins and arachidonate metabolites [3]

Generally, the increased use of HPLC procedures for the purification and separation of eicosanoids reflects (a) their easy optimization and reproducibility, (b) their selectivity for closely related metabolites such as the HETEs, which are difficult to resolve by GC procedures, even on high-efficiency capillary columns, (c) the advantages of being able to perform the separations at room temperature, sparing thermally labile compounds, and (d) the possibility of concurrently analysing compounds with a wide range of polarities and structural complexities, such as the PGs, C_{20} hydroxy acids and leukotrienes However, one of the problems of the HPLC determination of these compounds is to quantify them at their occasionally extremely low physiological levels Although HPLC systems are compatible with almost any type of on-line chromatographic detector, including the highly sensitive fluorescence and electrochemical detectors, the limits of detection for eicosanoids are usually unsatisfactory as these compounds in themselves are neither good chromophores, nor fluorophores, nor sufficiently electroactive Thus, for instance, in the assay of eicosanoids there is no equivalent in performance to the HPLC–electrochemical detection (ED) systems so successfully used for the high-sensitivity detection of biogenic amines [4] in biological samples Consequently, most of the high-sensitivity determinations of eicosanoids in biological extracts have traditionally been carried out by GC–MS or direct RIA and occasionally by HPLC after subjecting the samples to appropriate derivatization procedures in order to render them amenable to spectrophotometric detection

Fig 1 Arachidonic acid metabolic cascade as given by K C Nicolaou and N A Petasis, in P B Curtis-Prior (Editor), *Prostaglandins Biology and Chemistry of Prostaglandins and Related Eicosanoids*, Churchill Livingstone, Edinburgh, 1988, p 79 (Reprinted by permission)

Recently, with the advent of efficient thermospray interfaces for direct coupling of HPLC column effluents to mass spectrometers, we have been able to report on a few applications in the field of eicosanoids [5,6] Nevertheless, in some instances, the limits of detection are still inadequate even though they

can be substantially improved by suitable derivatization of the samples [6] On the other hand, as fraction collection of HPLC eluates is simple and can be readily automated, this facilitates the alternative use of sensitive 'off-line' detection systems such as bioassay, mass spectrometry, scintillation counting and radioimmunoassay

RIA, like MS, can be very sensitive and, further, it is readily available in most laboratories so that it has become the most widely used method for the routine quantification of eicosanoids However, the commercial availability and relative technical simplicity of the assay procedure make it especially prone to use by scientists unaware of possible sources of error and experimental pitfalls [7] As a result, the literature has been cluttered with widely varying values, which, in many instances, are not even physiologically realistic It is nowadays well known that many of the erroneous reports on PG determinations were due to excessive and unwarranted confidence in the specificity of RIA, disregarding the many factors, not only immunological but also non-immunological in nature, which can interfere with the binding of the antigen by the antibody These factors have been discussed in some detail by Granstrom and Kindhal [8]

2 CONTRIBUTIONS OF HPLC TO RIA

Whereas RIA as a means of detection can lend HPLC its very high sensitivity for the quantification of eluates that would not otherwise be detected, HPLC can provide a wider coverage to RIA procedures in (a) the initial phases of development of immunoassay methods by purification to homogeneity of the antigenic compounds needed for preparing hapten–protein conjugates, (b) the validation of direct RIAs and (c) the isolation of purified antigens and/or tracers for RIA determinations

When raising antibodies against specific antigens, the purer the latter the higher is the specificity of the assay Thus, when necessary, HPLC may contribute to ensure the homogeneity of a given antigen for its coupling to a suitable carrier protein or polypeptide. The resulting conjugate is then injected into an experimental animal in order to raise the proper antibody The same could be said for the isolation and purification of tracer compounds for RIA, as shown in the literature [9,10] Also, as with any other analytical method, HPLC can be used to validate results obtained by direct RIA of an analyte in a biological sample [11], provided that the analyte levels are high enough for detection and quantification by standard on-line HPLC detectors

Nevertheless, validation of RIA results is commonly performed by combined isotope dilution GC–MS techniques on account of their higher specificity and sensitivity Finally, eluent collection at the outlet of the HPLC column to isolate selected analytes for their subsequent determination by off-line RIA is the most common approach to increase the assay specificity and will be considered

here in some detail In this regard, the HPLC–RIA procedures are commonly based on three well defined and independent steps First, the samples need to be enriched in the class of substances that are to be analysed (in this instance eicosanoids) by removing salts, proteins, lipids, etc (sample clean-up) Next, the fraction enriched in the components of interest must be further purified so that ideally each of them is isolated from the remainder in a pure form (HPLC purification) Finally, the presumably pure collected HPLC fractions, after removal of the HPLC eluent, are quantified by standard immunological means (RIA procedures)

3 SAMPLE CLEAN-UP

Sample clean-up by the application of liquid- or solid-phase extraction procedures for the enrichment of eicosanoids in biological samples has been extensively applied prior to subjecting the samples to RIA. Reasons in favour and possible drawbacks have been discussed by Granstrom and Kindhal [8], who concluded that extraction may not always be necessary and that it even may introduce more problems than it solves

Obviously, amongst the reasons for extraction one could mention the concentration of analyte and removal of those interfering compounds capable of influencing the results of an RIA determination, and the drawbacks can be exemplified by the lower capacity of the method, possible procedural losses, introduction of interfering factors derived from the columns and/or the solvents used and emulsion formation in liquid–liquid extraction In general, the sample clean-up procedures most favoured at present for minimizing some of these problems involve the use of reversed-phase cartridges or mini-columns

Basically, as described by Powell [12], fatty acids, PGs and polar PG and fatty acid or arachidonate metabolites can be extracted from aqueous media using adsorption by reversed-phase ODS silica followed by elution of selected fractions with normal-phase eluents Relatively clean extracts with sufficiently high recoveries are thus obtained and emulsion formation is avoided In this system, the samples, properly acidified (pH 3–3 5) to suppress the ionization of the eicosanoid acidic groups in order to enhance retention on the reversed-phase materials, are loaded on to the mini-columns in a suitable solvent Elution with dilute aqueous ethanol followed by light petroleum–chloroform mixtures recovers polar lipids and fatty acids (HETEs) Prostanoids can then be eluted with ethyl acetate, methanol or methyl formate Finally, moderately polar hydrophilic substances can be recovered in 80% ethanol. The method has become very popular and in fact many of the references from the past four years on HPLC–RIA procedures for eicosanoids describe the use of Powell's method (or modifications of it) on Sep-Pak C_{18} cartridges prior to the HPLC isolation of selected fractions for their quantification by RIA In general, solid-phase extraction of biological fluids and tissue homogenates before

HPLC or RIA achieves the necessary removal of proteins and salts, which are washed away prior to sample elution

While extraction on solid-phase cartridges is still used to obtain enriched fractions for direct RIA [13], it is usually acknowledged that this is not sufficient in many instances as non-specific interferences may still yield spurious results [9] In some instances, the overall specificity of the Sep-Pak procedure can be improved by using a more specific elution sequence to wash away the interferents, as shown by Heavey et al [14] In this example, non-specific immunoreactivity in the HPLC–RIA for LTE_4 after Sep-Pak extraction can be reduced below the RIA limit of detection by washing the cartridge with acetic acid, light petroleum and chloroform before eluting the peak of LTE_4-like immunoreactivity with methanol

In some instances, eicosanoids are extracted on normal-phase silicic acid columns These are eluted, for instance, with mixtures of toluene–ethyl acetate [15] for PG recovery or with a sequence of solvents of increasing polarity as described by Mayer et al [16] They first extracted the incubation medium used for endothelial cells into ethyl acetate and applied the dry extract dissolved in diethyl ether–light petroleum to a column of acidic silica, which was eluted in sequence with diethyl ether–light petroleum (25 75 for free acids and neutral lipids and 75 25 for monohydroxy fatty acids) and ethyl acetate–methanol (90 10 for PGs and 50 50 for LTs and phospholipids) In a sense, this is similar to the elution sequence of Powell [12], where a series of increasingly polar organic solvents are used on reversed-phase column

As with any sample extraction and purification process, solid-phase extraction is not exempt from quantitative losses, which occasionally can be substantial, depending on the type of packing, type of compound adsorbed, volume of eluting solvent and solvent system used, as shown recently in the literature for the isolation of LTs from plasma samples [17] or when combined with further purification by HPLC For instance, in one example recoveries of PGs and thromboxanes after Sep-Pak extraction ranged from 73 to 90%, but decreased to 31–52% after an additional HPLC step [18] However, despite some discrepancies in the literature regarding recoveries, many reports to date show almost quantitative recoveries (80–100%) by solid phase extraction on C_{18} reversed-phase or Amberlite XAD-type resins, although if not properly optimized the recoveries can be as low as 10% [17] In this regard, and as illustrated in Table 1 showing composite data from different assays recently carried out in our laboratory, the sample matrix on which the eicosanoids are incorporated may also have a definite effect on extraction recoveries Whereas all of the values in Table 1 fall within an acceptable range, those from pancreas are 6–10% lower on average than recoveries from urine and nasal secretions Also, in our hands, a comparative appraisal of several commercial brands of octadecyl reversed-phase extraction cartridges yielded excellent recoveries (90%) for both HETEs and LTs, with minor variations which can be corre-

TABLE 1

RECOVERIES OF PROSTAGLANDINS AND THROMBOXANE IN DIFFERENT TYPES OF BIOLOGICAL SAMPLES

Recoveries were obtained by loading Sep-Pak C_{18} cartridges with a known volume of sample (1 ml of pancreas homogenate, 5 ml of urine and 2 5 ml of nasal lavage) supplemented with a known amount (ca 50 00 dpm per sample) of ^{3}H-labelled standards Prostanoids were eluted with methyl formate as in ref 12 These fractions were then taken to dryness under vacuum and reconstituted in HPLC buffer (ca 200 μl) A 50% aliquot was counted on a β-counter to calculate the recoveries of each individual compound The remainder was injected into the HPLC column (see Table 2) n = Number of assays

Compound	Recovery (%)		
	Pancreas ($n=8$)	Urine ($n=6$)	Nasal lavage ($n=8$)
6-Keto-$PGF_{1\alpha}$	83 5±2 2	93 0±2 0	92 0±3 4
TXB_2	81 0±2 5	95 7±4 1	89 6±4 5
PGE_2	87 5±3 9	93 7±3 2	90 1±2 7

lated to the carbon loading on the silica support and weight of material in the cartridge These observations will be published in detail elsewhere Other comparative studies on the use of solid-phase extraction columns for lipoxygenase metabolites are also available [17, 19], together with examples on how the whole sample clean-up procedure can be efficiently automated using a precolumn method [20] In this way, the sample is first injected into the precolumn (acting as the solid-phase extractor) and, after an adequate washing procedure to elute all unretained interferents out of the system thought the waste line, the solvent flow is diverted to the analytical column and the retained compounds of interest are eluted from the precolumn into the analytical column, where they can be separated and collected in the normal fashion

4 HIGH-PERFORMANCE LIQUID COLUMN CHROMATOGRAPHIC PROCEDURES

There are many reported HPLC methods for the separation of eicosanoids in biological fluids and tissues In general, acidic eicosanoids are well separated at low pH, where the ionization of the carboxyl groups is suppressed so that migration is based on lipophilic characteristics This is one of the strong points of HPLC as it is capable of resolving closely related structures with minor or subtle differences in hydrophobicity such as the isomeric PGE_2 and PGD_2 or the isomeric HETEs

Invariably, with very few exceptions [21], all of the work carried out in this field makes use of the power of reversed-phase columns and all those applications involving the use of HPLC as a separation system to purify compounds

for subsequent quantification by RIA are no exception The advantage of reversed-phase over normal-phase HPLC systems is that whereas the latter are capable of resolving most PGs and hydroxy fatty acids of interest, some of the LTs do not elute from these columns Also, the reversed-phase systems, as with the extraction cartridges, accept direct injection of aqueous samples and this makes them fully compatible with the aqueous nature of biological sample matrices.

The only restriction that RIA imposes on the HPLC system is that the eluents must be compatible with the immunoassay procedure In other words, they must be volatile so that the collected fractions can be readily evaporated to dryness and redissolved in the appropriate RIA buffers. Also, on drying there must not be any residue left (e.g., involatile buffer or ion-pair reagents) that could interfere with the characteristics of the antigen–antibody binding In this regard, the most typical eluent system for the HPLC separation of eicosanoids in RIA determinations is methanol–water–acetic acid with the pH adjusted within the range 5–6 and in proportions around 70 30 0 1 (see Table 3) In some applications acetonitrile is used in place of methanol and, in general, runs are isocratic except when a wide range of polarities must be covered.

However, with these solvent systems compounds such as thromboxane B_2 (TXB_2) elute as very broad and tailing peaks [22, 23] so that they cannot be collected in a single narrow fraction for RIA This effect can be prevented using an eluent of water with 0 04 M formic acid adjusted to pH 3 15 with triethylamine–acetonitrile (65 35) [24], as shown in Fig 2b Nevertheless, depending

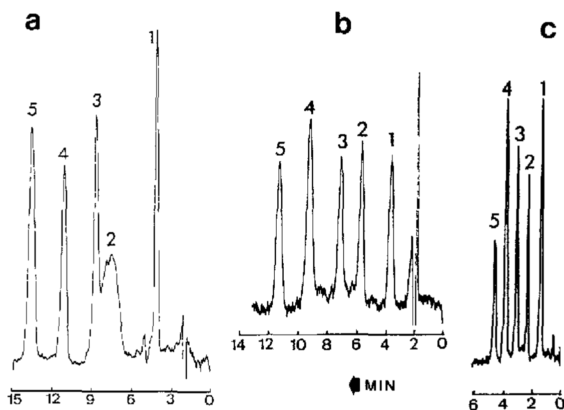

Fig 2 (a) HPLC profile of prostanoids separated on a 300 mm × 3 9 mm I D column packed with 10-μm Spherisorb ODS-2 with water with acetic acid to pH 3 4–acetonitrile (65 30, v/v) as eluent (b) Same as (a), except for the eluent, which was 0 04 M formic acid with triethylamine at pH 3 15–acetonitrile (68 32, v/v) (c) Same as (b) except for the use of a high-speed 100 mm × 4 6 mm I D column packed with 5-μm particles UV detection at 198 nm Peaks 1 = 6-keto-$PGF_{1\alpha}$, 2 = TXB_2, 3 = $PGF_{2\alpha}$, 4 = PGE_2, 5 = PGD_2

on minor deviations in pH adjustment and/or variations in the physical condition of the stationary phase due, for instance, to irreversibly adsorbed compounds from previous runs, the elution profile of TXB_2 is not always satisfactory, even under these conditions [25] A similar problem was also observed with the PGI_2 metabolite, 2,3-dinor-6-keto-$PGF_{1\alpha}$, which elutes as a very wide and deformed peak [26] In both instances, the problem lies in the coexistence of different equilibrium forms of the compounds depending on the pH of the medium Thus, by careful adjustment of eluent pH to 5 8 with TEA–acetic acid, it was possible to obtain an HPLC separation suitable for quantitative RIA work [27] In general, losses of sample components due to irreversible adsorption or changes in molecular conformation within the HPLC column are a definite possibility for prostaglandins, so that even though extraction recoveries prior to HPLC may be over 80% (Table I) they may drop to less than half in the combined extraction (Sep-Pak)–HPLC procedure, as indicated above [18] Table 2 gives the recoveries obtained after subjecting the extracts referred to in Table 1 to a further HPLC purification which, according to these data, results in an additional 10% loss on average Also, in our experience, a step in the process where eicosanoid losses are often overlooked is that of evaporation of the mobile phase in the HPLC fraction in order to reconstitute the sample in the proper RIA buffer Although spontaneous evaporation at room temperature has been recommended [8], this is not practical when a large number of samples in aqueous media have to be processed Thus, reduced-pressure evaporation is the preferred procedure in these instances In this regard we have recently established that under standard lyophilization conditions the absolute recoveries of 6-keto-$PGF_{1\alpha}$, TXB_2 and PGE_2 were 87 0 ± 2 6, 87 3 ± 2 4 and 89 0 ± 3 3%, respectively (mean ± S D , $n=5$) Nevertheless, in most instances, all of these losses are more than compensated for by the increased selectivity of the RIA when this is performed on purified HPLC fractions, as will be discussed below

A further experimental observation that should be taken into account when

TABLE 2

COMBINED SOLID-PHASE EXTRACTION AND HPLC RECOVERIES OF PROSTAGLANDINS AND THROMBOXANE IN DIFFERENT TYPES OF BIOLOGICAL SAMPLES

Recoveries were calculated directly from the counts in each of the HPLC fractions corresponding to the retention times of the three prostanoids Sep-Pak C_{18} extraction of the samples preceded the HPLC purification as indicated in Table 1 n = Number of assays

Compound	Recovery (%)		
	Pancreas ($n=8$)	Urine ($n=6$)	Nasal lavage ($n=8$)
6-Keto-$PGF_{1\alpha}$	70 6 ± 3 8	82 2 ± 3 8	88 9 ± 4 2
TXB_2	69 4 ± 5 2	84 2 ± 4 3	76 7 ± 9 0
PGE_2	78 1 ± 5 3	85 2 ± 5 2	84 8 ± 3 5

collecting fractions from an HPLC run for subsequent RIA and which can affect recoveries is that the retention times of labelled and endogenous compounds may differ by as much as 5% [9] with the labelled material eluting slightly ahead Thus, the setting of the collection window for the component of interest must allow for such differences A good common practice is to establish retention time windows for the analytes of interest with radioactively labelled standards of high specific activity which can be readily detected with flow-through HPLC radioactive monitors In this way one avoids injecting the large amounts required for the UV detection of cold analytes, which may interfere with the RIA blanks For example, in the RIA determination of 2,3-dinor-6-keto-$PGF_{1\alpha}$ in urine [27], where there was no labelled standard available, the optimal retention window for eluate collection had to be established by injection of a large amount (900 ng) of unlabelled material, as shown in Fig 3a In contrast, only low picogram (30–40 pg) amounts of labelled PGs need to be injected to establish their corresponding retention time windows, as shown in Fig 3b With real samples, the precise retention time window for 2,3-dinor-6-keto-$PGF_{1\alpha}$ is established relative to the retention time of a small amount of labelled PGD_2 which is injected, for instance, every tenth run to control

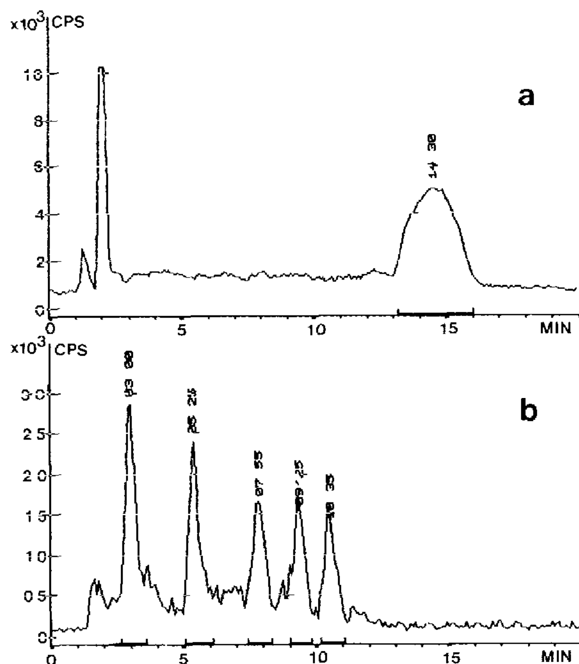

Fig 3 (a) HPLC separation of 900 ng of ^{3}H-labelled 2,3-dinor-6-keto-$PGF_{1\alpha}$ injected under the same conditions as in Fig 2b (b) HPLC separation of 30–40 pg amounts of ^{3}H-labelled 6-keto-$PGF_{1\alpha}$, TXB_2, PGF_2, PGE_2 and PGD_2 Same conditions as in Fig 2b Radiometric detection

small shifts in the elution profile Also, as shown in Fig 2c, the definition of proper collection windows becomes really critical when using high-speed HPLC columns because the peak widths are much narrower relative to those characteristic of conventional HPLC columns On the other hand, the resulting lower fraction volumes can be evaporated much faster For practical purposes, only 5-μm particle HPLC packings are used for faster elution, as real high-speed separations on 3-μm materials would be critical to control as regards eluate collection efficiency Also, injection volumes must be kept small, the smaller is the particle size, and this may affect the overall sensitivity of the assay

5 RIA OF SELECTED HPLC ELUATE FRACTIONS

Briefly, RIA procedures involve the prior inoculation of suitable animals with the desired antigen in order to raise the necessary antibodies for the assay In the usual practice, the sample extracts together with the radioactively labelled antigen tracer synthesized for this purpose are incubated overnight with the diluted antiserum obtained from the immunized animals The antigen in the extract binds the antibody, displacing the tracer as the assay is based on the competition between tracer and antigen for the binding sites in the antibody raised against the antigen The tracer is displaced in proportion to the amounts of antigen present in the sample extract, thus increasing the radioactivity of the unbound fraction The bound and unbound fractions are separated by the polyethylene glycol, charcoal or double-antibody methods and the amount of antigen is obtained by comparison with calibration graphs [8,28]

There is abundant information in the literature on RIA performance and procedures, as the method dates from 1959 and has been used extensively in different biomedical fields A recent appraisal of practices and pitfalls in RIA was published by Yalow [28], who concluded that RIA is being used in thousands of laboratories for all kinds of peptidic and non-peptidic compounds Undoubtedly, the introduction of many commercial assays has played a major role in the popularity of the method, even though they are generally expensive However, the alternative to commercial RIA kits requires access to the necessary animal facilities plus synthesis expertise to obtain the labelled ligands

The foremost advantage of RIA methods lies in their high sample capacities and remarkably high sensitivities, together with good serological specificity, even though in many instances the latter is clearly questionable This is where HPLC may contribute significantly to the reliability of a given assay by prior purification of the selected antigen to almost or full homogeneity

A recent literature survey showed that a number of laboratories use antisera supplied by collaborating institutions whereas others raise antisera specifically tailored to their problems Further, many of the latter even synthesize the labelled antigens or obtain them biosynthetically through an appropriate enzymatic reaction (see, for instance, refs 9–11, 14, 27 and 29) To obtain anti-

sera against eicosanoids, these arachidonic acid metabolites, by virtue of their small size limiting their antigenic characteristics, must be rendered antigenic through coupling to suitable carrier proteins or polypeptides, the so-called hapten–protein conjugates. In general, this is carried out by using the free carboxyl group of the eicosanoids and the amino groups on the protein as coupling sites. A thorough evaluation of the practice of developing an antibody for RIA is available in the literature [8] In any case, the selection of coupling sites is of foremost importance for the sensitivity and serological specificity of the resulting antibodies Accordingly, if the carboxyl group on the eicosanoid is used for coupling, the antibodies raised against the conjugate will recognize the ring and/or ω-end of the molecule However, with metabolites with a dioic acid moiety, conjugation may take place at either carboxyl group so that the resulting antibody will be non-specific for either of the two possible structures, unless one of the carboxyls is blocked in order to obtain a more homogeneous conjugate

A classical example reported by Granstrom and Kindahl [8] is illustrated in Fig 4 The development of an RIA method for the main urinary metabolite of $PGF_{2\alpha}$, $5\alpha,7\alpha$-dihydroxy-11-ketotetranorprosta-1,16-dioic acid, is facili-

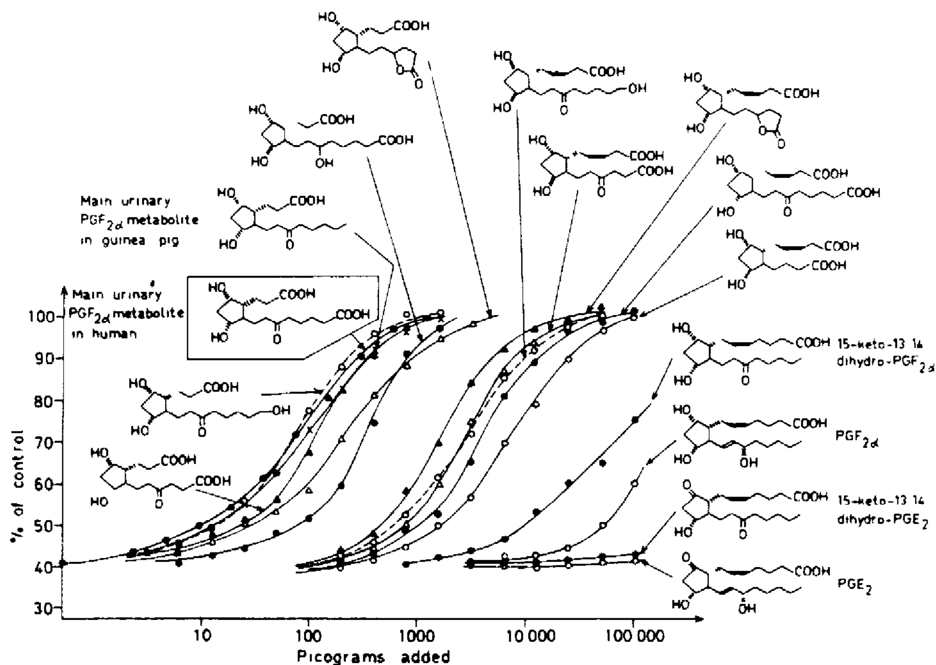

Fig 4 Properties of an antiserum raised against a conjugate of bovine serum albumin and the ω-carboxyl group of $5\alpha,7\alpha$-dihydroxy-11-ketotetranorprosta-1,16-dioic acid (the α-carboxyl group was protected by δ-lactone formation) as described in ref 8 (Reprinted by permission)

tated by the ready lactonization between the C-1 carboxyl and the 5-OH groups in acidic media so that coupling would take place at the ω-end, as deduced from the curves in Fig 4 The group of curves to the left indicate that the antibody cross-reacts to a high degree with other tetranor compounds and that the structure at the ω-end is irrelevant regarding its contribution to the extent of binding affinities for these compounds The central curves show that dinor metabolites are also recognized, although to a smaller extent, and finally the cross-reactivities with the parent C_{20} prostaglandins are minimal.

On the other hand, as shown by the curves in Fig 4, lack of absolute specificity might be an advantage for group-type assays of isolated HPLC fractions with a single antibody, as we have also recently reported [27] for the determination in human urine samples of 2,3-dinor-6-keto-$PGF_{1\alpha}$, a metabolite of prostacyclin (PGI_2) for which there was no antisera available In this instance, one of the antisera prepared against another PGI_2 metabolite, 6-keto-$PGF_{1\alpha}$, showed sufficient cross-reactivity to allow its use in the assay of HPLC fractions collected at the retention time of the dinor metabolite This approach greatly simplified the determination of 2,3-dinor-6-keto-$PGF_{1\alpha}$ in the urine of cirrhotics with portal hypertension (PH) and ascites [30], as illustrated in Fig 5 An updated account of the physiological implications of the higher levels of this metabolite in these patients as opposed to controls has been presented recently [31] Briefly, the increased systemic production of PGI_2 in cirrhotics with PH was associated with haemodynamic changes but the suppression of PH does not modify the systemic production of PGI_2, as indicated by the levels of the dinor metabolite This approach has also been used by other workers,

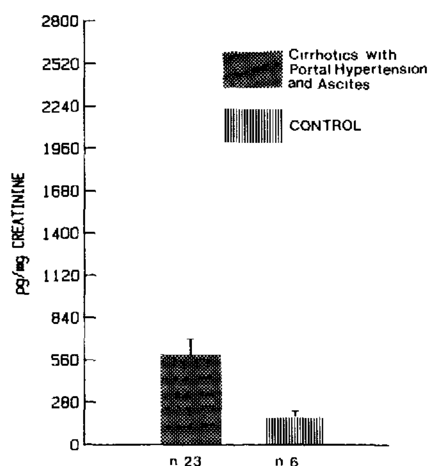

Fig 5 Levels of 2,3-dinor-6-keto-$PGF_{1\alpha}$ in cirrhotics and in controls as determined by RIA of HPLC-purified fractions with an antiserum raised against 6-keto-$PGF_{1\alpha}$ [27]

for instance for measuring PGE_3 with a PGE_2 antibody cross-reacting to 50% [22]

Commonly, RIA procedures involve the use of tracers labelled with a long-half-life β-emitter such as tritium, even though the specific activity or a ^{3}H-labelled compound and consequently the assay sensitivity are much lower than those which can be attained with a ^{125}I-labelled tracer However, with ^{125}I it is difficult to ensure the stability of labelled products and its short half-life severely limits its shelf-life so that, for practical purposes, virtually all reports describing the use of RIA for eicosanoid determinations employ ^{3}H-labelled tracers, with some exceptions such as the work by Maclouf et al [32] or, more recently, that of Cox et al [11] on the determination (15*R*)-15-methyl-PGE_2 in which they iodinated the histamide derivative of the ligand

As indicated above, whenever the labelled ligand or tracer is not commercially available or when its cost may be prohibitive, a reasonable alternative is to obtain it through biosynthesis starting from the labelled parent This has been described, for instance, for the synthesis of [^{3}H]-9α,11β-$PGF_{2\alpha}$ [9] from [^{3}H]PGD_2 using purified PGF synthetase from bovine lung If this is not feasible, a possible solution would be to use a heterologous tracer with high cross-reactivity

6 SOURCES OF EXPERIMENTAL ERRORS

Critical considerations regarding the performance of an RIA method have been discussed in some detail in the literature [8, 28] and therefore we shall only consider here those performance factors related to eicosanoid determination in biological samples

Lack of serological specificity or excessive cross-reactivity of an antibody can occasionally be an advantage for the determination of the total production of a given compound using a single antibody capable of recognizing all closely related metabolites or, as indicated above, when the antibody specific against that compound is not available but the compound can nevertheless be determined with a cross-reacting antibody after its chromatographic isolation Despite these specific applications, excessive cross-reactivity most often becomes one of the main drawbacks of immunological methods and has to be circumvented by appropriate sample extraction and purification steps

The percentage of cross-reactivity of a given antibody towards other related compounds is usually stated in the publications involving RIA determinations and, although in a few instances the data are comprehensive, more often only the cross-reactivities of the more closely related compounds are given together with a statement indicating that cross-reactivities of all other compounds were, for example, $<1\%$ However, in many instances the data are not only limited but also not truly meaningful, as they refer only to readily available off-the-shelf compounds and do not take into account all of those metabolites possibly

present in a biological sample with antigenic determinants that may also be recognized by the antibody. Even with known compounds of relatively low cross-reactivity the situation could be critical, depending on their relative amounts in the sample. For instance, in an RIA for LTB_4 in platelets which also form large amounts of 12-HETE, the accuracy of the results may be affected by the latter even though its cross-reactivity in the assay is only 2% [33]. Hence it is always convenient to keep cross-reactions at a level <0.1%. This may be especially difficult in the field of eicosanoids because many of these compounds are structurally very similar, such as PGs of the first and second series, which differ only in a double bond. On the other hand, this situation should not be a limiting factor provided that the components to be quantified have been separated by HPLC prior to their RIA evaluation. Nevertheless, despite the high selectivity of HPLC, this is no absolute guarantee of success, as illustrated by a recent report by Fogh et al. [34]. In this instance LTB_4 from psoriatic skin lesions was separated by extraction on Sep-Pak C_{18} cartridges and further purified by reversed-phase HPLC. It was shown that the peak of LTB_4 coeluted with that of 5(*S*,12*S*)-di-HETE so that the value of LTB_4 in the samples was best expressed as LTB_4 equivalents measured by a chemokinesis assay, even though the cross-reactivity of the antibody against LTB_4 was only of 0.14% for 5(*S*,12*S*)-di-HETE. Interferences of HETE compounds were also noted by Bazan et al. [35] in the RIA of LTC_4 separated by HPLC.

A further consideration of importance in the RIA of HPLC eluates is the carryover of standards used to determine retention time windows for fraction collection. As mentioned above, this is why it is best to inject very small amounts of labelled standards to delineate the appropriate time zones for collection of analytes. In any event, the injection systems and columns need to be properly washed before injection of the real sample, which should always be preceded by a procedural blank in which the elution zone of interest is checked for residual immunoactivity.

In some instances erroneous values are not due to interferences from other compounds but to artefactually raised levels consequent on preanalytical factors associated to the sampling process itself, as discussed in some detail in the recent literature [36]. For example, with regard to measurements of TXB_2 as an index of thromboxane production in vivo and even though normal plasma basal levels are in the 2–3 pg/ml range, there are many discrepant reports placing this value around 50–100 pg/ml. The problem can be circumvented by monitoring a circulating metabolite that cannot be artefactually generated [10]. For this purpose, 11-dehydro-TXB_2 was selected as a more reliable parameter of thromboxane production. This work also raises an interesting point in that, as a function of pH, the dehydrogenated metabolite can occur in two forms with different physico-chemical properties (ring δ-lactone and acyclic forms), so that antibodies against 11-dehydro-TXB_2 may recognize any of the two forms

differently For this reason and as the immunological methods are usually carried out at physiological pH, all standards and samples must be incubated for sufficient time to ensure complete hydrolysis of the lactone to the open form, which is the stable configuration at this pH [10]

Still, in other instances interferences may arise from non-immunological sources generating a number of factors that are capable of disturbing the ligand antibody binding These factors are usually related to pH variations, protein content or ionic strength Along these lines, data have been presented on the inhibition of antigen–antibody binding in the RIA of a $PGF_{2\alpha}$ metabolite due to release of interfering material (probably free fatty acids) from the plasma albumin in the course of protein precipitation with acetone As a result, basal levels of the metabolite increased to a level about three to four times those considered normal [37] The same effect may be observed when samples are solid phase-extracted because of impurities eluted from the columns [7, 8] Also, the same group has recently discussed how certain batches of heparin give rise to increased levels of monitored PGs if used in relatively large amounts either in vivo or in vitro to prevent blood clotting [7]

Finally, we have also experienced some problems related to non-specific binding and interferences in RIA determinations of prostaglandins (PGE_2, TXB_2 and 6-keto-$PGF_{1\alpha}$) in rat urine Briefly, when the appropriate labelled antigen or tracer was added to rat urine samples (5 ml) at a dilution of 1 7 without antibody, the binding of the antigen was as high as 96% of the maximum binding obtained with antibody This could be explained by different mechanisms For instance, the urine samples could contain compounds capable of binding the antigen, although perhaps the most likely explanation would be that as the free and bound fractions were separated by precipitation with dextran-coated charcoal, the charcoal could become saturated with interfering compounds present in the urine so that free tracer would remain in the supernatant and it could be counted together with the bound antigen This would be an effect opposite to the adsorption of both tracer and antigen–antibody complexes when there is insufficient carrier protein to saturate charcoal binding sites for the complexes These observations are of interest for the negative consequences these phenomena may have on the accuracy of the values reported for a given RIA Thus, at a dilution of 1 7 the values obtained for these prostanoids were much lower than those in the literature, whereas at 1 24 dilution these values became normal The effect was not encountered, however, when the same procedure was applied to human urine samples In this instance it must be taken into account that 5 ml of rat or human urine correspond to 1/4 or 1/500 of the total 24-h urine volume, respectively, so that the concentration in rat urine would be higher and in fact it has been experimentally verified that rat urine is less clean than human urine

7 VALIDATION OF RIA RESULTS

Considering all of the above facts, immunoassays would need to be properly validated to ensure the reliability of results For this purpose there are several options open that range from the very simple tests of inhibition with aspirin or other anti-inflammatory drugs to the use of sophisticated equipment, such as combined GC–MS techniques In terms of specificity and sensitivity, the latter is indeed the best method to check for the specificity of a given RIA Depending on the type of sample and the compound(s) monitored, the correlation between RIA and GC–MS values may be 0 9, with good agreement with actual values, as recently shown for LTB_4 in human neutrophil samples [38] or for 6-keto-$PGF_{1\alpha}$ and TXB_2 in breast tumours [15] However, although these comparisons may uncover a good correlation, the actual values may be higher than those obtained by GC–MS, as reported for instance for 5-HETE in a solid phase (Sep-Pak)-extracted sample of a lung extract, where the RIA values were 70% higher [39] This proves the contribution of interfering components in the sample, as further demonstrated by the fact that these high values attained levels closer to those obtained by GC–MS after an additional purification by HPLC

Further or alternative validation of the accuracy of an RIA procedure can be obtained from simple tests such as the parallelism test, whereby samples subjected to various dilutions must give RIA plots parallel to that of the standard For consistency of results, samples should be always assayed at at least two dilutions

Also, the response from samples treated with enzymatic inhibitors should drop to values close to zero if there is no contribution from non-specific binding This is illustrated in the example in Fig 6, where the basal levels of TXB_2 and PGE_2 generated by cultured monocytes were reduced to values close to zero in the presence of indomethacin On the other hand, the concentration of both compounds increased, as would be expected, after stimulation of the cells with zymosan [40] In another example, the amounts of eicosanoids formed by isolated rat glomeruli were reduced by 90% with BW755C, a dual cyclooxygenase and lipoxygenase enzyme inhibitor However, the concentration of LTB_4 in particular was reduced by only 44%, confirming that not more than 25% of the initial immunoreactive material was LTB_4 The remainder was not recovered at the HPLC elution window of LTB_4 [41] Alternatively, the RIA method should also respond to the addition of known amounts of the monitored compound to specimens pretreated or not with enzymatic inhibitors or with a controlled diet suppressing this compound [22]

On the other hand, as stated above, HPLC may also serve as a complementary method to validate the accuracy of an RIA determination In these instances, samples may be quantified by measuring the integrated absorbance with a UV detector at the column outlet, and these values can then be com-

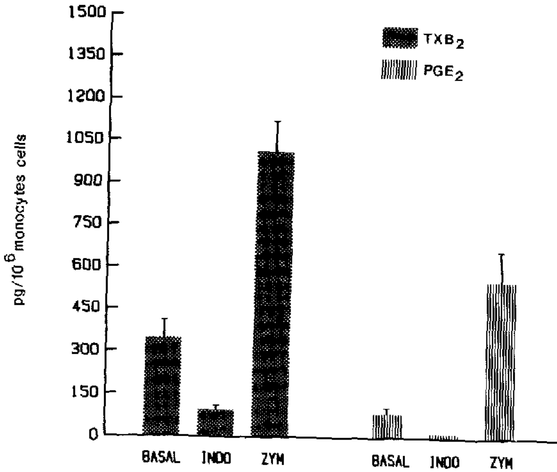

Fig 6 RIA levels of TXB_2 and PGE_2 in monocyte cell incubates [40] INDO = indomethacin, ZYM = zymosan

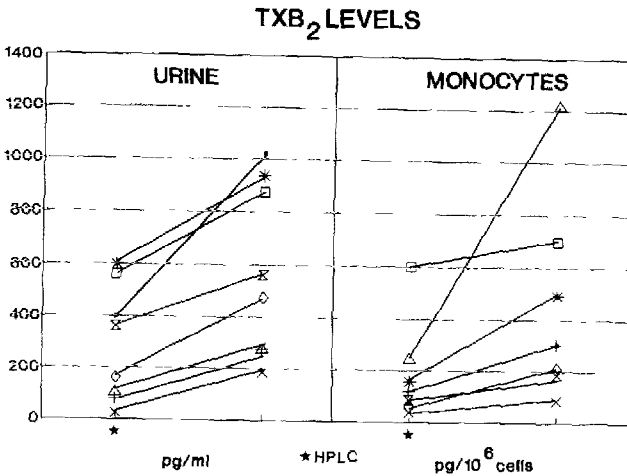

Fig 7 TXB_2 levels in urine and monocyte cell incubates as determined by direct RIA of solid-phase extracts or by RIA of the same fractions after additional purification by HPLC (★)

pared directly with immunoreactivity data [11, 34, 42, 43] Recent data from our laboratory on the production of TXB_2 by monocyte cells in culture [40] and on the concentration of this prostanoid in urine samples shows that RIA values are always lower if obtained on HPLC-purified fractions (see Fig 7) In one instance the difference observed between direct RIA and RIA with HPLC-purified fractions is very noticeable (from about 250 to over 1200 pg per 10^6 cells), pointing to interferences in the direct assay However, depending on the type of sample, in other instances, the values may agree much better, as

shown, for example, in a study [44] on the role of PGE_1 in attenuating pulmonary oedema provoked by hydrogen peroxide In this instance, both RIA and HPLC analysis detected increased amounts of 5-HETE in the perfusion medium of hydrogen peroxide-injured lungs (RIA, 48 0 ± 14 7 ng; HPLC, 54 8 ± 13 5 ng) relative to controls (RIA, 6 6 ± 1 6 ng, HPLC, 6 8 ± 1 9 ng) The increase was reduced with PGE_1 (RIA, 29 2 ± 8 3 ng, HPLC, 29.8 ± 7.6 ng) These data show that the excellent correlation between HPLC and independent RIA values proves that the latter are free from the effect of interferences.

Also, taking into consideration that, as already discussed, HPLC purification of extracts, while providing fractions containing mostly pure isolated compounds, does not ensure that this is always the case, the accuracy of results could be greatly improved by collecting the fractions in two different HPLC systems In practice this is not usually done, but by running the extract under two different eluent and/or column systems and monitoring the fractions with the same RIA one could rely on the absence of interferences from coeluting antigenically active compounds if the RIA values were to agree in the two sets of fractions

On the other hand, for all practical purposes and unless one is specifically interested in accurate absolute values, the results of RIA studies showing poor accuracy (e g , higher values) but good correlations with those obtained with a validating technique can still be useful in a biomedical setting. This is because in many instances the answers sought from immunoassays are relative changes occurring in quantitative values after certain manipulations or pharmacological treatment of the specimens

8 LIMITS OF DETECTION

Low limits of detection (the smallest amount of substance assayed that can be measured) are one of the principal assets of RIA which, if properly performed, can be extremely sensitive for certain substances in biological samples In fact, the sensitivity of immunological methods is only matched by negative-ion selected-ion monitoring GC–MS methods Usually, detection limits are reported in the low picogram range, although they can even be much lower, especially when using ^{125}I-labelled tracers of very high specific activity. For instance, the sensitivity of RIA determinations of 6-keto-$PGF_{1\alpha}$, TXB_2 and PGE_2 in plasma of diabetics carried out with a commercial ^{125}I RIA kit was reported to be 0 2, 0 7 and 0 02 pg/ml, respectively [45] The sensitivity of the assay was defined in this instance as twice the standard deviation at zero binding A useful and at the same time informative way of defining the practical limit of detection for quantification purposes is as given by Cox et al [11], the amount of analyte that gives a response n times greater than the intra-subject standard deviation of the sample background

In ultimate terms it could also be said that considerations regarding detec-

tion limits are what dictates, in addition to interfering factors already discussed, the requirement for preconcentration of the compounds to be quantified Thus, whenever the concentration of a component to be measured in a sample is below the detection limit of the available RIA kit, the sample needs to be extracted and the component concentrated [36] by any possible means, including HPLC purification

9 SELECTED APPLICATIONS OF HPLC-RIA PROCEDURES

The data in Table 3 give a general overview of the most recent applications of RIA applied to solid phase-extracted and HPLC-purified fractions Unfortunately, the literature search on which this review is based also uncovered a few interesting applications which do not provide sufficient information on the experimental approach to allow their inclusion As shown, applications are entered by sample type and compounds identified by RIA with a brief summary of the methods used for solid-phase extraction and subsequent HPLC purification of the solid-phase extracts This table can be considered to be representative of the work reported in the most recent literature within the last four to five years As can be seen, with the exception of a single case [35], all of these applications involve a solid-phase extraction on Sep-Pak-type cartridges with recoveries that in many instances are >90% (see Section 3) Powell's elution method [12] for eicosanoids or modifications are usually encountered, methanol–ethyl acetate or methyl formate being the most commonly used solvents to recover the eicosanoids retained by these cartridges Further purification by HPLC is carried out on octadecylsilane reversed-phase columns with mobile phases which are RIA-compatible after evaporation The most commonly used mixture in this regard is composed of methanol or acetonitrile, water and acetic acid in an isocratic mode, although a few applications resort to gradient runs [21, 33, 46]

10 ENZYME IMMUNOASSAYS AS ALTERNATIVE NON-RADIOACTIVE METHODS

The HPLC-purified antigens can also be determined by alternative non-radioactive immunological methods based on the monitoring of the products arising from enzymatic reactions The enzyme immunoassays (EIAs) not only avoid all of the problems related to the use of radioactive material, such as instability of labelled products and cost of their isolation and disposal, but also should allow the determination of radioactive ligands, for instance in metabolic tracer studies, which would not be possible by RIA Several EIA methods have been described for various PGs and also for thromboxane and LTs, all of them providing data of quality comparable to those obtained by RIA EIA is dependent on the competition between enzyme-labelled and unlabelled antigens for the binding sites of a suitable antibody followed by the assay of enzyme

TABLE 3

COMBINED HPLC-RIA PROCEDURES FOR VARIOUS TYPES OF SAMPLES

Sample	Solid-phase extraction[a]		HPLC purification[a]			Yield (%)	RIA	Ref
	Wash	Elution	Column packing[b]	Mobile phase	pH			
Serum	EtOH-H_2O (1 9)	MeOH	1	(A) MeOH-H_2O (65 35), (B) MeOH-H_2O (68 32), A→B	6 5 5 6	– –	LTB_4 LTC_4	33
Blood, lymph	H_2O-hexane	MeOH	1	MeOH-H_2O-AcA (65 35 0 08)	5 5	–	LTs	42
Blood	Toluene-EA-MeOH Toluene-EA	Toluene-EA-MeOH	2	ACN-H_2O-AcA (33 67 0 1)	–	–	TXB_2, 6-keto-$PGF_{1\alpha}$	15
Blood	AcA-light petroleum-$CHCl_3$	MeOH	5	MeOH-AcA (0 05%)	5 6	52	LTE_4	14
Blood	H_2O	MeOH-AcA	1	ACN 0 1%-AcA (36 64→50 50)	–	–	LTC_4	46
Skin fluid	MeOH-H_2O	H_2O-MeOH	1	ACN-H_2O-AcA (40 60 0 1)	5 8	–	LTC_4, LTD_4	47
Skin biopsies	MeOH-H_2O (pH 3)	H_2O-MeOH-light petroleum	3	MeOH-H_2O-AcA (70 30 0 1)	–	–	LTB_4, 15-HETE	34
Human milk	Powell's modification	Me formate	6	ACN-H_2O+PO_4 (32 68)	3 0	30-36	PGE_2, $PGF_{2\alpha}$ $DHKF_{2\alpha}$	48
PMN incubates	Acetate buffer (5 6)-H_2O	MeOH	2	MeOH-H_2O-AcA (60 40 0 06)	–		LTC_4	49
Eosinophil incubate		MeOH	2	MeOH-H_2O-AcA (70 30 0 1)	5 4	–	LTB_4, LTC_4	50
Lung incubates	5% Acetic acid	MeOH	4	MeOH-H_2O-AcA (67 33 0 08)	6 2	70	LTs	51
Lung perfusates	H_2O-$CHCl_3$	MeOH	3	MeOH-H_2O-AcA (72 28 0 16)	4 9	40-80	LTs	43
	H_2O	MeOH	9	MeOH-H_2O-H_3PO_4 (30 70 0 02), gradient to 100% MeOH	5 7	73	LTC_4	52

Nasal washes	Powell's modification	Me formate		ACN-H_2O-AcA (35 65 0 2)	3 4	85–90	$PGF_{2\alpha}$, PGE_2	53,54
Nematodes	11 1% MeOH-light petroleum	MeOH	2	MeOH-H_2O-AcA (70 30 0 1)	5 4	–	LTB_4, LTC_4	55
Fly extracts	Powell's	Light petroleum-Me formate	1	TEA-F-ACN (69 31)	–	–	PGE_2	29
Rat glomeruli	Powell's	EA	4	MeOH-H_2O-AcA (65 35 0 01)	5 7	75	LTB_4	41
Brain homogenate	Powell's	EA	7	ACN-H_2O-AcA (30 70 0 01)	–	25–40	$9\alpha,11\beta$ $PGF_{2\alpha}$, $PGF_{2\alpha}$	9
Kidney cortex	H_2O-EtOH-light petroleum Benzene	EA-MeOH (9 1) ACN-MeOH (10 1)	8	H_2O-ACN-benz Ac (76 7 23 0 2 0 1)	–	27–37	PGE_3	22
Urine	Powell's		1	AcA-TEA-ACN (68 32)	5 8	–	Dinor 6 k $F_{2\alpha}$ TXB_2	27
Urine	H_2O-light petroleum	Me formate	1	ACN-H_2O-AcA (67 37)	3 4	–	PGS	56
Frog retina	None							
	Liquid-liquid extraction	$CHCl_3$-MeOH	1	ACN-MeOH-H_2O-AcA (403 65 532 10)	5 6	–	LTC_4, LTD_4	35

[a]EtOH, ethanol, MeOH, methanol, Me, methyl, EA, ethyl acetate, AcA, acetic acid, ACN acetonitrile, TEA, triethylamine, F, formic acid, Powell's, Powell's solid extraction method [12]

[b]1, μBondapak, 2, Nucleosil, 5 μm, 3, Hypersil ODS, 5 μm, 4, Spherisorb ODS, 5 μm, 5, Ultrasil ODS, 6, Novapak, 7, Cosmosil 5 C_{18}, 8, C_{18} fatty acid analysis, 9, μPorasil

activity When the enzyme-labelled antibody is immobilized on a solid-phase support, to simplify the separation of antibody free from antibody-bound antigen, the assays are known as enzyme-linked immunosorbent assays (ELISAs), as opposed to precipitation methods for EIAs Basically, the experimental procedures of an EIA developed, for instance, for TXB_2 involve the following steps (a) preparation of the antibody as for RIA (conjugation of TXB_2 to a suitable protein and immunization of animals with subsequent collection and storage of serum), (b) coupling of TXB_2 to the enzyme β-galactosidase, (c) binding of the antibody to a polystyrene tube by incubating for 1 h at 37°C; (d) addition of sample solution and enzyme-labelled TXB_2, (e) incubation and washing of the tube, and (f) addition of a suitable enzyme substrate such as 4-methylumbelliferyl-β-D-galactoside and measurement of the fluorescence of the resulting product, 4-methylumbelliferone [57] More recently, the same basic procedure has been described by the same authors for 13,14-dihydro-15-keto-$PGF_{2\alpha}$ in human blood [58]

In both instances the antigens to be monitored had been previously purified by HPLC and in terms of detectability both RIA and EIA were comparable and in the low femtomole range Likewise, satisfactory correlations were obtained between EIA and RIA ($r \geqslant 0$ 9) and between EIA and GC–MS As happens in RIA with the specific activity of radioactively labelled tracers and affinity of the antibody, the ultimate sensitivities of the EIA methods depend on the use of enzymes of high specific activity, such as acetylcholine esterase, which has been reported to provide EIA sensitivities equivalent to, or better than, RIA [59]

Good correlation of RIA and solid-phase EIA was also reported in a study on the basal levels of PGD_2 in rat brain [60] Sample preparation for EIA and RIA included solid-phase extraction on ODS cartridges and reversed-phase HPLC In this instance PGD_2 was labelled with horseradish peroxidase (HRP) The anti-PGD_2 antibody was coated on polystyrene, twelve-well, flat-bottomed microstrips PGD_2-HRP and free PGD_2 in the samples competed in solution for a fixed number of antibody sites on the wells The proportion of PGD_2-HRP bound to the antibody was reduced as the concentration of free PGD_2 increased, with the result that after appropriate washing procedures to separate antibody-bound from free PGD_2 tracer the measured enzyme activity on the wells decreased In this instance, 3-(p-hydroxyphenyl)propionic acid and hydrogen peroxide were used as substrates to monitor the activity of the enzyme

As the antibody cross-reacted to a significant extent with PGD_1 and PGD_2, these interferences were effectively suppressed by the HPLC separation Thus, as indicated by the authors, with the aid of the HPLC purification the EIA for PGD_2 can detect amounts as low as 3 pg per assay

The development of EIAs can also make use of the double- or triple-antibody methods, as described in the preparation of an ELISA for LTs [61] In this

instance, rabbit anti-LTC_4 antiserum was coated with goat anti-rabbit globulin linked to fluorescein isothiocyanate (FITC) and then a third antibody, rabbit anti-FITC globulin linked to alkaline phosphatase was added The substrate was a solution of *p*-nitrophenyl phosphate and, as the activity of adsorbed alkaline phosphatase was much higher than that of the other antibodies, the overall sensitivity was much improved

11 PERSPECTIVES IN BIOMEDICAL APPLICATIONS

From the data available in the literature, it seems that RIA methods have contributed greatly to furthering our knowledge in the field of eicosanoid metabolism and function in a wide diversity of biomedical applications Also, it is evident that the potential of the technique has been definitely strengthened by the application of highly efficient HPLC purification techniques and solid-phase extraction procedures All of these techniques are capable of compensating for the lack of specificity of many RIA procedures, especially when applied to a family of closely related compounds With these considerations in mind, it becomes evident that liquid chromatographic purification prior to RIA will not only continue to be used but also that it will become a routine procedure in many applications Nevertheless, on account of all of the shortcomings associated with the use of radioactively labelled material, in many instances the RIA methods will be replaced by EIAs although these still need to be properly standardized for practical purposes Likewise, one of the main challenges of immunoassay methods in general lies in the full automation of the chromatographic purification In this regard robotics and laboratory information management systems will undoubtedly play a major role in the near future and will contribute to reducing the overall costs of these assays In this regard, nowadays it is well proved that solid-phase extraction, injection into HPLC columns and collection of eluate fractions are steps readily amenable to fully automatic performance Further, the technology for automatic operation of various of the immunoassay steps, such as pipetting, diluting, dispensing, scintillation counting and reporting, is also commercially available so that a fully integrated automatic system should not be too far off in the future

12 SUMMARY

An evaluation of the most recent literature on the determination of eicosanoids by immunoassay methods confirms that owing to the inherent lack of specificity of many of the antibodies used for this purpose, immunological assays (radioimmunoassay or enzyme immunoassay) are often preceded by solid-phase extraction followed by further purification of the antigens of interest by routine reversed-phase high-performance liquid chromatographic methods In

thıs way the analytıcal potentıal of radıoımmunoassay ıs remarkably enhanced and accuracy and precısıon of the assay are ensured

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