Journal of Pharmaceutical & Biomedical Analysis
Vol. 5, No. 8, pp. 759–765, 1987
Printed in Great Britain

0731–7085/87 $3.00 + 0.00

Quality control of antibodies with special reference to prostaglandins*

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Abstract: Sources of error in the immunoassay of prostaglandins are reviewed. First, the specificity of the antibody, in terms of cross-reactions with structurally related substances, is often tested against irrelevant compounds, i.e. available compounds that do not occur in the biological material under study; major metabolites occurring in much larger amounts are overlooked. Hence, the reported high specificity of some antibodies may be apparent only. Second, many eicosanoids occur in two or more chemical forms in equilibrium in aqueous media. Antibodies may recognize one form preferentially; thus comparison of data from different laboratories is difficult.

In addition numerous factors may interfere with the antigen–antibody binding in a non-immunological way. The most common effect is inhibition of antigen–antibody binding, but enhancement of binding sometimes occurs.

Keywords: *Antibodies; prostaglandins; radioimmunoassay; eicosanoids; quality control.*

Introduction

The prostaglandins constitute a large family of oxygenated C_{20} fatty acids of almost ubiquitous occurrence in mammalian tissues. The members of this family display a wide spectrum of biological effects, often with extremely high potencies. Consequently they have been implicated as modulators in numerous biological phenomena and as mediators of many pathological processes. To confirm such involvement, a great deal of effort has gone into the development of quantitative methods for determination of these compounds in various biological materials.

The most commonly employed quantitative assay for prostanoids is radioimmunoassay [reviews, 1, 2]. Today, antisera against many of the prostanoids and their major metabolites and derivatives are commercially available. Because of the relative simplicity of the method, it is unfortunately often used by scientists whose knowledge about underlying events is insufficient and who are unaware of the many sources of error that may be involved. It is thus not surprising that numerous reports with unrealistic data, obtained by radioimmunoassay, have been published on prostanoids.

*Presented at the "International Symposium on Immunoassays", November 1986, Arlanda, Sweden.

For example, basal plasma levels of certain prostanoids have been (and occasionally still are) reported in the nM range, although careful metabolic studies indicate that the compounds either do not occur in the circulation at all, or at most exist in concentrations of a few pM [1]. This was, for example, the case with prostaglandins of the A type in the early and mid-1970s. At that time, PGA was believed to be an important blood pressure regulating, circulating hormone. Many radioimmunological studies reported on how the blood levels of PGA varied with the patient's blood pressure, salt intake, posture, etc. Later developed, highly specific mass spectrometric methods demonstrated, however, that all these measurements were wrong, and that PGA is hardly detectable in the circulation. The compound is now regarded as an artifact [1].

If at all recognized, these artifactually elevated levels were usually explained as the result of cross-reactions with structurally related prostanoids. However, most readings were so high that unrealistic amounts of other prostaglandins would have been required; such amounts would not be present under normal conditions.

It is far more likely that such high "prostaglandin" levels were caused by other factors. Discrepancies in data reported from different laboratories are commonly seen, particularly when data are based on methods employing different antibodies and assay conditions. Comparison of data is thus difficult. In recent years, a great deal of effort has gone into standardisation of prostaglandin assays, including quality control of the antibodies employed.

Some major and common sources of error in prostanoid immunoassay are discussed in this review; these errors pertain mainly to the properties of the antibodies employed.

Specificity of the Antibody Employed

An obvious source of error in radioimmunoassays is cross-reaction between the antibody and one or more compounds that are not only structurally related to the monitored one, but also occur in the same sample in sufficient concentrations to interfere. Thus a thorough metabolic knowledge of prostanoids is necessary. A great number of different compounds (prostaglandins, thromboxanes, leukotrienes and related substances) originate in the same polyunsaturated precursor fatty acid and may be formed simultaneously by the biological system under study. Furthermore, most tissues possess metabolizing enzymes and rapidly degrade the prostanoids formed to varying degrees.

Monitoring a single, specific compound may thus be difficult if its degradation products or other prostanoids dominate the metabolite profile. Such attempts call for highly specific antibodies. Commercial antibodies are always supplied together with information about their cross-reactions with a large number of compounds; unfortunately, however, it seems that such lists are more often compiled from the particular compounds that happened to be available for testing than from those compounds that are biologically and chemically relevant.

Prostanoid antisera are generally raised against immunogenic conjugates, prepared by coupling the prostanoid carboxyl to amino groups on a carrier protein (Fig. 1). Thus, resulting antibodies normally do not recognize the carboxyl end of the hapten but are highly specific for the central (ring) part and the ω end. This fact has at least two important consequences:

(1) Cross-reactions with β-oxidized compounds (dinor and tetranor metabolites) may be considerable for an antibody against a C_{20} compound; conversely, an antibody raised against a dinor metabolite may also recognize the parent compound. As was briefly

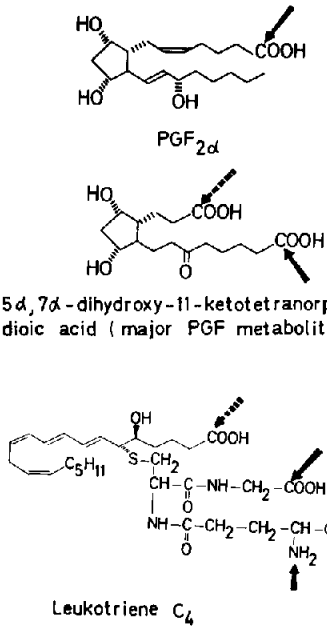

Figure 1
Common sites of coupling of eicosanoids to carrier proteins (generally to amino groups on the protein). Dotted arrows indicate where coupling is less likely to take place due to facile δ-lactonization during conjugation.

mentioned above, metabolites of different oxidation levels may coexist in a biological sample. In urine, for example, dinor and tetranor prostaglandins occur in concentrations two or three orders of magnitude higher than those of the parent prostaglandin. Cross-reactions with such C_{18} and C_{16} compounds are almost never reported; such information is only provided for a number of more or less randomly chosen C_{20} compounds. Since cross-reactions with substances altered in other parts of the molecule are often <0.01%, the information provided may deceptively give the impression of a highly specific antibody.

(2) Compounds containing more than one carboxyl group may be coupled at one or more sites other than the intended one (cf. Fig. 1), with quite different properties of the antibodies as a result. Unfortunately, with commercial antibodies it is not uncommon that information about the employed coupling method is not available; thus conclusions about the resulting antibody cannot be drawn.

Unsatisfactory specificity of the employed antibody need not be a draw-back, however. An antibody that cross-reacts extensively with many structurally related metabolites may provide reliable information on the total production of a certain compound. For example, antibodies against one cysteinyl-containing leukotriene (Fig. 1) often recognize the others as well (LTCs, Ds and Es). Since these display relatively rapid interconversion in biological material, monitoring of only one may give misleading information. Should specific measurement of one compound be necessary, however, the problem of low antibody specificity may be circumvented by purification of the samples.

Occurrence of Several Chemical Forms of the Eicosanoids

Many eiocosanoids can occur in more than one chemical form in aqueous media. For example, compounds with a hydroxyl group *delta* to the carboxyl are prone to lactonization. This is seen with tetranor metabolites of PGF compounds, with 11-

dehydrogenated thromboxanes, and with most products of the 5-lipoxygenase pathway, such as the leukotrienes (Fig. 2). Other compounds may undergo other types of reactions: 6-keto-$PGF_{1\alpha}$ may exist in an open and a lactol form; thromboxanes of the B-type similarly may have a closed hemiacetal central ring or exist in the open form; finally, prostaglandins of the E and D type may form epimers in respect of the side-chains, due to enolization of the keto groups (Fig. 2).

The occurrence of several chemical forms in equilibrium may be without significance for quantitation if the radioimmunoassay employed measures all forms of the particular compound with equal accuracy, or if the conditions of the assay lead mainly to the formation of one of the forms. Under other circumstances, however, the simultaneous existence of several forms of a compound may cause great discrepancies in assay results. A set of standards cannot be expected automatically to behave identically with biological samples, for example; furthermore, comparison of data from different laboratories becomes difficult.

A related problem is the irreversible decomposition of certain prostanoids in aqueous media. Several of these compounds are rather unstable and may rapidly loose water and form various dehydration products [2]. This is, for example, the case with PGEs and PGDs, whose half-lives under common radioimmunoassay conditions may be relatively short. The proportion of dehydration products formed may vary under different conditions. Consequently, the final readings of a radioimmunoassay that is supposed to measure "PGE" or "PGD" will depend substantially on the conditions of the assay and particularly the properties of the antibody.

Since coupling of the hapten to a carrier protein normally takes place during dehydrating conditions, the resulting antibody may preferentially recognize the

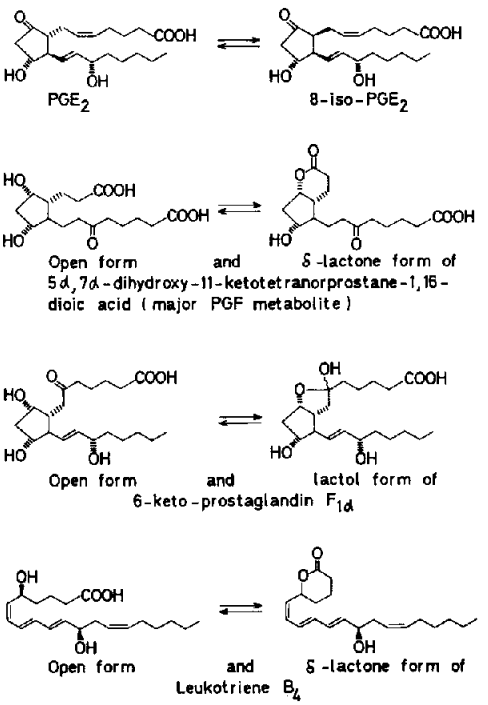

Figure 2
Occurrence of different chemical forms of eicosanoids in equilibrium in aqueous media.

degradation products. This was the case with the earliest developed "PGE" radioimmunoassays, which were directed exclusively at the corresponding PGA or B compound [1]. Similar difficulties explain the paucity of radioimmunoassays for PGDs [2].

Similar problems are encountered with leukotrienes. The peptido-leukotrienes (LTCs, Ds and Es, cf. Fig. 1), as well as some other lipoxygenase products, may isomerize at the Δ^{11} *cis* double bond to form 11-*trans* LTs. This isomerization may have taken place already during the preparation of the immunogen [3]. The formation of oxidation products (sulphones, sulphoxides) of the cysteinyl containing leukotrienes should also be kept in mind [4].

When unstable compounds are monitored, it is sometimes preferable not to try to prevent degradation but instead to direct the assay at either a stable derivative or a degradation product and to induce this reaction to completion in the samples prior to assay [e.g. 5, 6].

Non-immunological Interference in Radioimmunoassay

The only raw data that a radioimmunoassay provides is information on the degree of inhibition between the antibody and the labeled ligand. This binding is easily disturbed by numerous factors totally unrelated to the monitored compound, such as variations in protein content, ionic strength and pH; this fact is not generally recognized. It is mandatory to keep in mind that samples and standards can never be assayed under completely identical conditions. This is unfortunately the case whether the samples are extensively purified or assayed directly as such.

A biological sample naturally contains numerous unrelated substances which could interfere. From this point of view it seems self-evident that samples should be purified prior to assay. A fact that is often overlooked is, however, that purification may enrich substances other than the monitored ones and may even introduce impurities. Different antibodies display variable sensitivities to interference by such substances due to differences in their avidity. This is another area where quality control of the employed antiserum is important but where information is often missing.

Most of the interfering substances cause decreased binding between antibody and antigen. If its true causes are overlooked, such decreased binding may thus be erroneously interpreted as high "prostaglandin" levels in the radioimmunoassay. Many processing procedures have revealed such interference. For example, the authors' radioimmunoassay for a major, circulating $PGF_{2\alpha}$ metabolite, 15-keto-13,14-dihydro-$PGF_{2\alpha}$, is highly accurate when applied to small volumes of unextracted plasma [7]; basal levels are generally in the range of 100–200 pM, which is in agreement with data from mass spectrometric methods [8]. After a simple processing step, however, such as precipitation of plasma proteins with acetone followed by spontaneous evaporation of the solvent, the "basal" levels increased to about 600–700 pM [7]. Furthermore, these "prostaglandin" levels were not influenced by treatment of the subject with aspirin in doses known to give almost complete inhibition of the prostaglandin biosynthesis. However, if after processing, the samples were restored with respect to albumin, the basal levels were again in the normal range and responded to aspirin [7]. Thus, it is likely that interfering material was released from the plasma albumin when the proteins were precipitated with acetone, and that this material inhibited antigen–antibody binding in the radioimmunoassay. Part of this material may be free fatty acids. Such compounds

normally display very low cross-reactivity in most prostanoid radioimmunoassays (<0.01 or 0.001%) but the total concentration in normal plasma may be in the μM range. The solution to this problem is thus either to assay plasma samples without extraction or, if this is impossible, to purify them extensively after extraction, preferably by HPLC.

A problem related to this type of interference is when lipolytic drugs are used *in vivo*. Heparin is often administered systemically to maintain potency of intravascular catheters. Among other effects, heparin also causes liberation of free fatty acids, which may be recorded as increased levels of the monitored "prostaglandins" by immunoassays. The compound is also known to interfere directly with antigen–antibody binding in a similar way. Thus, it is not even safe to use heparin only *in vitro* for collection of plasma. If heparin must be used, the concentration should be as low as possible (around 10–25 IU ml^{-1} blood to avoid clotting). Recently, a new brand of plastic tubes has been used containing lithium–heparin in the authors' studies of bovine reproduction. Analysis of 15-ketodihydro-$PGF_{2\alpha}$ levels in plasma collected in these tubes showed "basal levels" about 10-fold higher than in plasma from heparinized (sodium–heparin) Vacutainer tubes (H. Kindahl *et al.*, unpublished observation); this "increase" was only an artifact, possibly caused by the particular batch of heparin. However, no other direct radioimmunoassay used in these studies was disturbed.

Another similar artifact is seen when the resin Amberlite XAD-2 is used for extraction of biological samples. Some unknown material from the resin, which cannot be removed by prior washing, strongly inhibits antigen–antibody binding. In the prostaglandin literature, reported levels are invariably unrealistically high and "resistant" to inhibition by aspirin, for example, when based on radioimmunoassay of XAD-2 processed samples.

In addition, other types of chromatographic procedures may be associated with the same kind of artifacts. Interfering impurities have been found in silicic acid chromatography and reversed-phase partition chromatography and read as high "prostaglandin" levels in a subsequent radioimmunoassay [9]. Evaporation of samples under a stream of nitrogen can also lead to falsely high "prostaglandin" levels when compared with

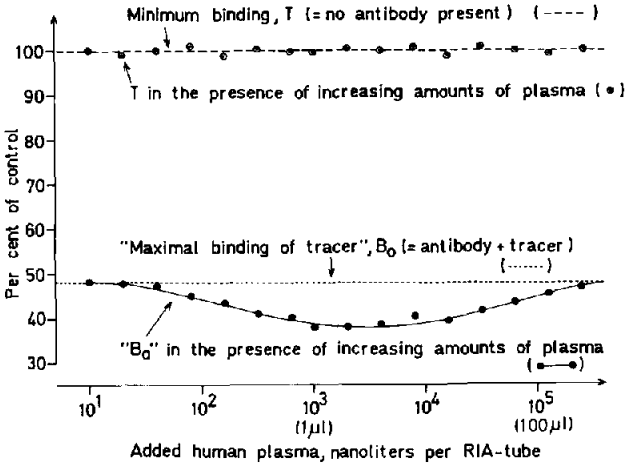

Figure 3
Synergism between an antibody against 11-dehydro-TXB_2 and one or more unknown components in plasma in binding of the labelled ligand. To solve this problem, a minimal amount of plasma had to be added to the entire set of standards as well.

spontaneously evaporated samples. The identity of the interfering substances is not known.

The opposite phenomenon, i.e. increased binding of the ligand, is less common but may be an even more serious source of error. It is seen with some antibody preparations and with biological material, particularly plasma, from some species, but may be totally absent from others. A typical example is shown in Fig. 3; an antibody against a thromboxane metabolite, 11-dehydrothromboxane B_2, interacts with one or more unknown components in plasma from several species to give a pronounced enhancement of the initial binding. As little as 1 μl of plasma is sufficient to induce the phenomenon maximally. Plasma itself is without effect (Fig. 3). The implication of this deleterious synergism is that if assayed directly, biological samples may fall completely below the standard curve. The problem may be solved in two different ways: by extensive purification of the samples prior to assay; or by inclusion of a small volume of plasma in all tubes, including the set of standards. Usually, the latter solution is preferred.

Acknowledgements: This study was supported by grants from the Swedish Medical Research Council (proj. no. 03X-05915) and from Petrus and Augusta Hedlund's Foundation.

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[*Received for review 21 January 1987*]