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Labelling of haptenic drug with digoxigenin for competitive immunoassay: its application to lesopitron, a new anxiolytic agent

Albert Adam[a,*], Jaime Rojas[a,1], José Pretel[b], Luis Martínez[b], Huy Ong[a]

[a]*Faculté de Pharmacie, Université de Montréal, Case Postale 6128, Succursale Centre-Ville, Montréal, Québec, H3C 3J7, Canada*
[b]*Laboratorios Dr. Esteve, SA, Department of Pharmacokinetics and Drug Metabolism, Barcelona, Spain*

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Abstract

A new labelling approach of haptenic drugs with digoxigenin for the development of competitive enzyme immunoassay (EIA) is reported. It consists of the covalent linking of the hapten to the preactivated digoxigenin derivative and revealing the immune complexes with anti-digoxigenin Fab fragments coupled to alkaline phosphatase. This approach has been applied to the development of an EIA for the pharmacokinetic study of lesopitron (E-4424), a new anxiolytic agent. The assay involves a solid-phase immobilization of IgG purified from polyclonal antiserum developed against the butylamino derivative of lesopitron covalently linked to bovine serum albumin. The tracer consists of the covalent linking of the same butylamino derivative to digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester. The calibration curve for E-4424 from 12.5 to 6400 pg per well displays an ED_{50} of 34.5 pg per well, a slope factor of 0.86 and a minimum detectable dose of 4.1 pg per well. The accuracy and the precision of the assay assessed at three different concentrations of E-4424 (500, 1000 and 2000 pg ml^{-1}) give a recovery higher than 95% and intra- and inter-assay RSDs lower than 5 and 10%, respectively. The specificity of the assay was demonstrated by a good correlation of the samples analysed by both HPLC and EIA. A kinetic profile of E-4424 in rats following an oral dose of 50 mg kg^{-1} has also been established.

Keywords: Digoxigenin; DIG–antiDIG system; Enzyme immunoassay; Lesopitron

1. Introduction

Radioimmunoassays which have been used extensively for half a century to quantify hormones and drugs in plasma raise some concern about the radioactive nature of the tracers. To overcome this drawback, non-isotopic labelling using enzymes and fluorescent and bio- and chemiluminescent molecules have been developed [1,2].

Among these non-isotopic systems, biotin and avidin derivatives are now commercially available. The avidin–biotin complexes have been exploited in solid-phase enzyme immunoassays

* Corresponding author. Tel.: (514) 343-2026; fax: (514) 343-2102.

[1] Present address: Departamento de Farmacia, Facultad de Ciencias, Universidad Nacional de Colombia, Santafé de Bogotà, Columbia SA.

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(EIAs) which involve biotinylation of antibodies, labelling of biotin or streptavidin with various enzymes and revealing the immune complexes with chromogenic, fluorescent and luminescent substrates [3–5]. The main disadvantage of the avidin–biotin system is its non-specific background due to the presence of biotin in biological samples of animal origin and to the molecular characteristics (alkaline p*I* and high hydrocarbon content) of the avidin [3]. More recently, different derivatives of digoxigenin, a cardenoline extracted from *Digitalis lanata*, have been developed by Boehringer Mannheim to label cDNA probes, proteins and peptides [6–8]. Taking into account the unique phytochemical origin of digoxigenin and the availability of anti-digoxigenin enzyme-labelled Fab fragments, the digoxigenin–anti-digoxigenin system (DIG–antiDIG system) appears to be a more specific indicator for mammalian biological samples than biotin–avidin [6].

We report in this paper the first application of digoxigenin in developing a tracer for a haptenic drug and the use of the DIG–antiDIG system for setting up a competitive EIA for a new anxiolytic compound, 2-{4-[4-(4-chloro-1-pyrazolyl)butyl]-1-piperazinyl}pyrimidine dihydrochloride (Lesopitron). Lesopitron is a centrally acting 5-hydroxytryptamine 1A agonist and appears to display anxiolytic properties [9–11].

2. Materials and methods

2.1. Materials

Sephadex G25M and Mabtrap G kit were purchased from Pharmacia (Uppsala, Sweden). Superfreeze was obtained from Pierce (Rockford, IL). Dimethylformamide (DMF) was purchased from Anachemia (Montreal, Quebec, Canada), *N*-methylmorpholine, Tris base, Tris–HCl, Tween 20, glutaraldehyde and *p*-nitrophenyl phosphate (pNPP) from Sigma (St. Louis, MO), digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (DIG-OSu), alkaline phosphatase-labelled Fab fragments (IgG) anti-digoxigenin and alkaline phosphatase (AP) of EIA grade from Boehringer Mannheim (Mannheim, Germany) and E-4424 (Lesopitron), E-5145 (the butylamino derivative of E-4424) and E-5043 (the 5-hydroxy-E-4424 metabolite) from Esteve (Barcelona, Spain). All other reagents, unless specified otherwise, were of analytical grade and purchased from Fisher Scientific (Fair Lawn, NJ). Sep-Pak C18 and an analytical HPLC system equipped with a UV detector were obtained from Waters (Milford, Ma, USA). A FRAC 100 collector and a beta counter were supplied by LKB (Bromma, Sweden).

A Speed Vac was obtained from Savant (Farmingdale, NY) and immunoplates A/C Nunc from Nunc (Roskilde, Denmark). A Behring ELISA processor (Behring Institute, Marburg, Germany) was used for the EIA step.

2.2. Methods

2.2.1. Development of a polyclonal antiserum anti E-4424

Polyclonal antiserum anti E-4424 was obtained in rabbits (Charles River, St.-Constant, Quebec, Canada) by immunization against E-5145 covalently coupled to BSA (100 μg per animal) using glutaraldehyde [12]. After five boostings, the rabbits were bled and the serum collected, diluted in glycerol (50%, v/v) and stored at −80°C. In a screening study, a radioimmunoassay (RIA) using [^{3}H]-E-4424 as a tracer allowed the selection of an antiserum characterized by a dissociation constant at equilibrium at 100 pM with a concentration of binding sites of 323 pmol l^{-1}. This antiserum exhibited no significant cross-reactivity (0.32%) with the 5-hydroxylated metabolite of E-4424. Polyclonal IgG was extracted from this antiserum using immunoaffinity for Protein G Sepharose (MabtrapG) according the recommendations of the manufacturer. The absorption at 280 nm of the purified material allowed the calculation of the concentration of IgG [13].

2.2.2. Obtaining tracers for the quantification of E-4424 by competitive EIA

2.2.2.1. Labelling of E-5145 with AP. This tracer was obtained following the coupling of E-5145 with AP using glutaraldehyde [14]. Briefly, 1.85 nmol of AP was diluted in 500 μl of a 100 mM phosphate buffer (pH 7.4) containing ZnSO4 (0.02 g l^{-1}) and dialysed against 2 l of the same phosphate buffer and 185 nmol of E-5145 and 1.85 μmol of glutaraldehyde were added to the AP dialysed solution. The mixture was then incubated for 1 h at room temperature, in the dark. The coupling was quenched by addition of 100 μl of 1 M glycine and was incubated for a further 1 h. The coupling medium was then transferred on to a Sephadex G25M column and the E-5145–AP derivative was separated from free E-5145 by elution with the phosphate buffer. The purified tracer was diluted in two volumes of Superfreeze and stored at −80°C.

2.2.2.2. Synthesis of an E-5145–DIG tracer. In a glass tube, 3 μmol of E-5145 and 1.5 μmol of DIG-O-Su were dissolved in 1 ml of dimethylformamide (DMF) alkalinized with 0.2 ml of *N*-methylmorpholine. The coupling reaction was performed following a 4 h incubation period at room temperature with stirring. The E-5145–DIG tracer was then selectively extracted on a Sep-Pak C18 cartridge previously activated with CH_3CN (5 ml) and primed with 0.1% trifluoroacetic acid (TFA) (10 ml). At the end of the incubation time, the coupling solution was diluted with 10 volumes of 0.1% TFA, loaded on a Sep-Pak C18 cartridge and then washed with a step gradient of 25%, 35% and 45% CH_3CN in TFA (0.1%). Each eluted fraction was analysed by HPLC. For the monitoring of the yield of the coupling reaction, the HPLC conditions consisted of a Vydac C18 column (Vydac, Hesperia, CA) and a linear gradient of CH_3CN from 10 to 90% in 0.1% TFA in 60 min at a flow rate of 1 ml min^{-1}. The absorbance was monitored at 240 nm.

2.2.3. Assessment of the immunoreactivity of E-5145–DIG complex

A 96-well plate was coated with 100 μl of purified polyclonal IgG (5 μg ml^{-1}) dissolved in carbonate buffer (100 mM, pH 9.5) for 24 h at 4°C. The uncoated material was then removed by a five-wash cycle using the incubation buffer (50 mM Tris–HCl, pH 7.4, containing 100 mM NaCl and 0.5 ml l^{-1} Tween 20). The plates were saturated with the same incubation buffer for 2 h at 37°C. The calibration solution (E-4424) or successives dilutions of E-5145–DIG complex (50 μl) and 50 μl of the tracer (E-5145–AP, dilution 1:100) were added to each well. The competitive immunoextraction step was completed in selected conditions, by incubation of the plates for 16 h at 4°C. After a further five-wash cycle, the enzyme activity bound to the immune complexes was detected by incubation (30 min, 37°C) in the presence of 100 μl of a 100 M diethanolamine buffer (pH 9.8) containing pNPP (1 mg ml^{-1}) and $MgCl_2$ (1 mmol l^{-1}). The absorbance was read at 405 nm using 490 nm as a reference wavelength.

2.2.4. Competitive EIA of E-4424 using DIG-labelled E-5145 as a tracer

In this case, immunoplates were coated with purified IgG diluted in the carbonate buffer, at a concentration of 430 ng ml^{-1}. After the different washing and saturation steps, the plates were incubated with 50 μl of the standard solution (E-4424: 3.1 to 6400 pg per well) or the plasma extract (50 μl) and E-5145–DIG (50 μl, diluted $1:3 \times 10^5$). The immunological reaction was achieved as described above. The unbound material was removed by a washing cycle and the bound E-5145–DIG was then allowed to react (2h, 37°C) with 100 μl of diluted (1:2000) anti-DIG Fab fragments labelled with AP in the incubation buffer. The enzyme activity of bound AP was finally revealed under the same conditions as described above.

2.2.5. Analytical validation of the competitive EIA

Plasma samples (1 ml) were diluted with 1 ml of Na_2HPO_4–citric acid (0.2 M, pH 6.8) buffer (McIlvaine buffer) and extracted with toluene (4 ml). After centrifugation, the organic phase (3.5 ml) was collected and evaporated in a Speed

Vac`system. The residue was resuspended in 500 μl of the incubation buffer before quantification in triplicate as described above.

The precision and the accuracy of the assay were assessed by testing blank serum spiked with E-4424 at three concentrations: 500, 1000 and 2000 pg ml^{-1}.

2.2.6. Kinetic profile of E-4424 in rat plasma

2.2.6.1. Animal protocol. Male Wilstar rats (Interfauna Spain) weighing 200–250 g were used. The animals were fasted for 16 h prior to dosing and 4–6 h post-dosing while water was allowed ad libitum throughout the study. They were housed individually in metabolic cages at 24 ± 2°C and a humidity of 55 ± 5% on a daily light/dark schedule of 12/12 h. The product was dissolved in water. A kinetic profile of E-4424 was set in a rat following an oral dose of 50 mg kg^{-1} of E-4424. Blood samples were collected after 15 min, 30 min and 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 24 h. E-4424 levels were determined following extraction by HPLC [15] and quantification by EIA as described above.

2.2.6.2. Blood sampling and E-4424 extraction. For plasma level determination, a small volume of blood was collected at selected times after administration of drug, then the plasma was separated by centrifugation at 3000 rpm for 10–15 min. Plasma concentrations of E-4424 were determined by a totally automated assay method based on a Prospekt on-line solid-phase extraction with an HPLC system with fluorescence detection [15]. Briefly, the extraction of E-4424 from plasma samples was carried out by using C2 Prospekt cartridges (10 × 2 mm i.d.) (Spark Holland, Netherlands). Sample (100 μl) was injected into the cartridge, then the cartridge was washed with H_2O and CH_3CN–H_2O (10:90, v/v) and E-4424 was eluted in the forward-flush mode with the mobile phase from the extraction cartridge on to the chromatographic column.

2.2.6.3. HPLC method. For the analytical separation, an ODS-2 Inertsil 5 μm column (250 × 4.6 mm i.d.) supplied by GI Sciences (Tokyo, Japan) was used in the reversed-phase mode. The mobile phase was KH_2PO_4 (20 mM, pH 3.0)–CH_3CN (78:22, v/v), the flow rate was 0.5 ml min^{-1} and the eluent was monitored by means of an HP1046A fluorescence detector (Hewlett-Packard) at an excitation wavelength of 235 nm and an emission wavelength of 385 nm.

3. Results

3.1. Synthesis and characterization of the E-5145–DIG tracer

3.1.1. Homogeneity of the tracer

The DIG-labelled E-5145 was eluted from the Sep-Pak cartridge with 35% acetonitrile in 0.1% TFA. The homogeneity of this fraction was assessed by HPLC, which displayed a unique peak eluted at 45% acetonitrile, corresponding to that of E-5145–DIG under the analytical HPLC conditions specified. Under such conditions, E-5145–DIG was separated from E-5145 and DIG-O-Su,

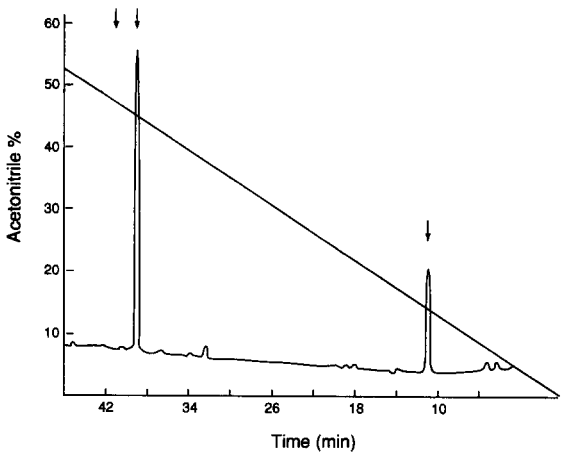

Fig. 1. Typical chromatogram of the incubation medium (10 μl diluted 1:1000 in 1% TFA) used for the synthesis of the E-5145–DIG tracer after the couping reaction has been completed. E-5145, DIGOSu and DIG-labelled E-5145 eluted by 14, 47 and 45% acetonitrile, respectively.

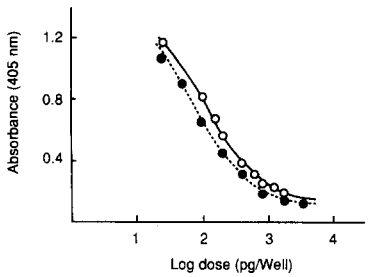

Fig. 2. Immunoreactivity of the E-5145–DIG derivative. Calibration curve for the competitive enzyme immunoassay of E-4424 using an alkaline phosphatase labelled tracer (◦) and displacement curve obtained with successive dilutions of the E-5145–DIG conjugate (•).

which eluted at 14 and 47% of acetonitrile, respectively (Fig. 1).

3.1.2. Immunoidentity of E-5145–DIG with E-4424

The immunoreactivity of the DIG derivative was tested in competitive EIA. Fig. 2 shows the displacement curves of the AP tracer by standard solutions of E-4424 and by successive dilutions of E-5145–DIG. The calibration curve ranges from 50 to 2400 pg per well. This curve is characterized by an ED_{50} of 155 pg per well (3.1 ng ml^{-1}), a slope factor of 1.22 and a minimum detection concentration of 29.4 pg per well (588 pg ml^{-1}). The slope factor (1.19) of the displacement curve obtained with successive dilutions of E-5145–DIG is not significantly different ($p < 0.001$) [16].

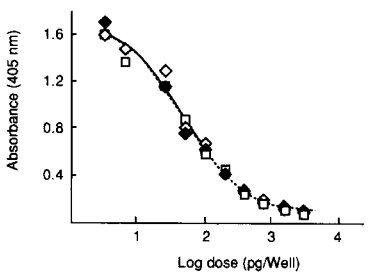

Fig. 3. Typical calibration curve for the competitive EIA of E-4424, using E-5145–DIG as a tracer, in the incubation buffer (◇) and spiked in a blank plasma extract (◆). A competition curve between E-5145–DIG and a plasma extract is also shown (□).

3.2. Competitive EIA using E-5145–DIG as a tracer

As shown in Fig. 3, a typical calibration curve for the EIA is characterized by a slope factor of 0.86, an ED_{50} of 34.5 pg per well (690 pg ml^{-1}) and a minimum detection level of 4.1 pg per well (82 pg ml^{-1}).

Under the same conditions of incubation, the calibration curve spiked with a blank serum extract was superimposable on the calibration curve for the buffer solution.

3.3. Analytical validation

The precision of the EIA was assessed by testing blank serum samples spiked with E-4424 at concentrations of 500, 1000 and 2000 pg ml^{-1}. These samples were analysed in five replicates over five different days. The intra-assay RSDs were 7.5, 6.7 and 2.4%, respectively, and the inter-assay RSDs were 8.8, 7.1 and 4.7%, respectively. The accuracy was tested using the same samples ($n = 5$); the recovery was higher than 95% in each case.

Similarly, the specificity of the assay was assessed by documenting the parallelism between the calibration curve and successive dilutions of a rat plasma extract containing the drug (1323 ng ml as determined by HPLC). The mean slope factors of both the calibration and dilution curves (0.93 and of 0.94, respectively) were not statistically different ($p < 0.001$) [16], confirming the identity of the extracted material as E-4424.

3.4. Application

Ten plasma samples of rat receiving 50 mg kg of E-4424 were analysed by HPLC and EIA. The correlation between these data could be described by the $y = 0.97x - 11.08$ ($r = 0.98$, $p < 0.001$).

The kinetic profile of Lesopitron measured in these samples by EIA is illustrated in Fig. 4. The mean serum concentration of E-4424 peaked at 1489 ng ml^{-1} (C_{max}) at about 30 min after the administration (T_{max}). Lesopitron could still be detected in the ng ml^{-1} range after 24 h.

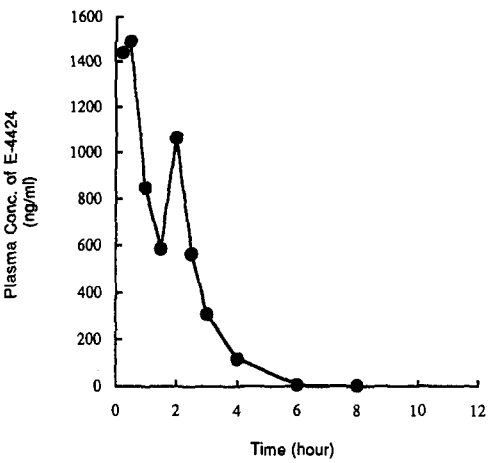

Fig. 4. Kinetic profile of E-4424 in a rat receiving 50 mg kg^{-1}.

4. Discussion

In the development of immunoassays, one of the critical steps is the labelling of the tracer. For peptides and proteins, the radioactive tracer is usually developed by labelling tyrosine or histidine residues within its structure with radioiodine. This approach is not suitable for the other organic substances, however. In EIA, the tracer resulting from the coupling of the haptens to enzymes raises concerns about the immunoreactivity of the haptens and about the enzymatic activity of the complex due to the denaturation resulting from the labelling procedure. We report here an interesting alternative for the labelling of the tracer by coupling preactivated digoxigenin derivatives to functional groups such as amino, sulphydryl or aldehyde commonly found in organic compounds. The stoichiometry of the conjugation step is well defined and the reaction is complete. Moreover, the covalently linked hapten to digoxigenin does not lead to a decrease in the affinity binding of the hapten to antibody in the competition reaction. Furthermore, the relationship of the immune complexes with anti-DIG alkaline phosphatase Fab fragments actually results in an increase in the sensitivity of the detection mode.

We have successfully applied this approach in developing a tracer for the EIA of Lesopitron, a 5-hydroxytryptamine 1A agonist which displays interesting anxiolytic properties and is rapidly metabolized through hydroxylation at position 5 of the pyrimidine ring. For pharmacokinetic studies of the compound, a sensitive EIA for the monitoring of the plasma levels of Lesopitron at subnanogram levels is therefore required. The butylamino derivative of E-4424 was selected for the synthesis of a specific polyclonal antiserum in rabbit which exhibits no significant cross-reactivity with the hydroxylated metabolite. In the same way, the coupling of the same butylamino derivative to the succinimide derivative of digoxigenin provides a suitable tracer for setting up a competitive immunoassay which allows the detection of Lesopitron at picogram levels. The assay has been validated in terms of accuracy with an overall recovery higher than 95% for Lesopitron spiked in plasma samples. It was also validated in terms of precision, giving an intra- and inter-assay RSDs lower than 10%. The specificity of the assay has been demonstrated by the correlation ($r = 0.98$) of plasma Lesopitron levels obtained both by HPLC and EIA.

In conclusion, the new analytical approach of labelling of organic compounds by covalent linking to digoxigenin and exploiting the DIG–antiDIG system in the development of EIA for small organic molecules offers interesting prospects in the development of EIA for monitoring plasma levels of synthetic organic drug compounds.

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