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Application of a heterogeneous immunoassay for the quality control testing of release-active forms of diclofenac

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ABSTRACT

We report on a specially designed diclofenac-ELISA for the determination of diclofenac in the presence of release-active forms of diclofenac in lactose dissolved in water solutions according to a predefined schedule in single-blind experiments. In accordance with the objective of this project, a number of experiments were conducted to determine the optimal ELISA conditions for detecting potential modulatory effects of release-active forms of diclofenac depending on their ability to affect the binding of diclofenac to anti-diclofenac antibodies. As a feature, the diclofenac antibodies were previously incubated with manufactured pharmaceutical samples containing release-active forms of diclofenac or placebo. For comparison of the sample types, measured in ELISA optical densities were chosen. For statistic analysis, Student's two-sample t-test and single-factor ANOVA were applied. The extremely low concentrations of diclofenac of 0.01, 0.05 and 0.1 ng mL^{-1} seem most appropriate for routine assay performance. The source of diclofenac used for standard solution preparation is not important but it could be important as the source of diclofenac for release active form of diclofenac preparation. As an outcome, the ELISA appeared to be suitable for the detection of the modifying effects of release-active forms of diclofenac toward the pharmaceutical substance in vitro.

1. Introduction

Starting from Samuel Hahnemann's pioneering work on homeopathy as a novel therapeutic method at the end of the 18th century, there has been an ongoing discussion of its reasonability [1–4]. The scientific community is split into opponents and proponents, each accentuating weak points of the counterpart, such as those relating to dosage of drugs at 'imaginary' concentrations or contempt for the individual character of homeopathic treatment, or results of meta-analysis provided by some clinical trials [5,6]. Despite the fact that a wide range of hypotheses has been postulated to justify and elucidate mechanisms of their action such as the theory of water memory [7–10], formation of clathrates [11], epitaxy [12], some hypotheses basing on the quantum physical aspects [1,3], etc., convincing evidence for this mode of action which would be based on rational studies involving modern physical and chemical methods is, although desirable, still missing [4,13,14]. The original technique to prepare the remedies consists of decreasing the initial concentration of a drug substance by multiple consecutive dilution or grinding (trituration) with lactose. In the 1990s, another concept called 'combined' or 'bipathic' treatment was introduced, which is based on the modifying effect of so-called 'release-active' (RA) forms of substances toward the initial pharmaceutical substance during their concomitant administration [15–17]. The efficacy of such RA therapeutics was demonstrated by using both biological (antibodies, cytokine, etc.) and chemical (prednisolone, phenazepam, etc.) compounds as the original substance [17–19]. One of the chemical substances under study is diclofenac-sodium. The ability of RA form of diclofenac to enhance the diclofenac efficacy and improve its safety has been already confirmed in previous in-vitro and in-vivo studies [16,20,21]. Thus, based on additive anti-inflammatory activity achieved by this co-administration one can suggest possible reduction in the dose of diclofenac in the future practice which may be useful to achieve the desired efficacy avoiding well-known gastric toxicities such as ulceration and subsequent hemorrhage [22–24]. Despite this fact, however, the issue of quality control of RA forms is currently on the agenda and the work on choosing the assay for quality control is ongoing. Generally, among the assays which are tested for the detection of high dilutions predominate assays which evaluate either some of its properties or effect-related influences. Among the examples of the first category are nuclear magnetic resonance [25,26], flux calorimetry, conductometry, pH-metry tests and galvanic cell electrode potential [8,27], ultraviolet spectroscopy [5,13,28,

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29], transmission electron microscopy, inductively coupled plasma-atomic emission spectroscopy [1], and other assays for electromagnetic property measurements [30,31]. Assays of the second category are commonly in vitro methods [2,4,6] but they may be used for recording some physical or chemical properties as well [32]. Because the ability to affect antigen–antibody interaction has been shown recently for this class of drugs [16], one of the possible approaches for quality control is using immunoassays.

Immunoassays are a high-sensitivity detection technology utilized in modern analytical laboratories. Originally applied in clinical chemistry, the related methods have become very popular in other fields like food and environmental chemistry over the last decades. In the authors' laboratory, a highly sensitive and specific indirect competitive enzyme-linked immunosorbent assay (ELISA) for the determination of diclofenac, a non-steroidal anti-inflammatory drug (NSAID), in water and biological samples was developed [33–35]. The aim of the present study was to use this ELISA technique for the quality control of RA forms of diclofenac preparations according to a predefined schedule in single-blind experiments. As a feature, the diclofenac antibodies were previously incubated with manufactured pharmaceutical samples containing RA form of diclofenac, placebo or standard. In accordance with the objective of this study a number of in-vitro experiments were conducted to determine the applicability and optimal conditions of a specially designed ELISA to detect possible modulatory effects of RA forms of diclofenac depending on their ability to the affect binding of diclofenac to anti-diclofenac antibodies. In five independent experiments, the optimal conditions for pre-incubation of anti-diclofenac antibody with experimental samples and for use of different diclofenac standard solutions in ELISA were identified.

2. Materials and methods

Experiments were performed at the Institute of Hydrochemistry and Chair of Analytical Chemistry, Technische Universität München (Munich, Germany). The polyclonal rabbit anti-diclofenac antibody was developed by our group [33]. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (H + L) (A6151), casein (C5890), ovalbumin (OVA; A5253), 3,3′,5,5′-tetramethylbenzidine (TMB; T2885), hydrogen peroxide 30% (95321), diclofenac sodium salt, D6899, (designated in this study as diclofenac '1'), and all standard chemicals and reagents were purchased from Sigma-Aldrich (Steinheim, Germany). For comparison, diclofenac sodium salt (designated as diclofenac '2') from Henan Dongtai Pharm Co. (China, 15307-79-6) was used. High binding 96-well polystyrene microplates (655061) were obtained from Greiner Bio-One (Frickenhausen, Germany). Buffers and solutions were prepared fresh in ultrapure water obtained from a Milli-RO 5 Plus, Milli-Q185 water purifying system (≥18 MΩ) (Millipore, Eschborn, Germany). Phosphate buffered saline (PBS) consisted of 0.01 M phosphate buffer solution and 0.137 M sodium chloride (pH 7.6).

2.1. Experimental samples

RA forms of diclofenac were supplied by Materia Medica Holding (MMH, Moscow, Russia) in a form of lactose(See Table 1). For lactose sample preparations, lactose was saturated with RA form of diclofenac on a 96 L fluidized-bed Pilotlab apparatus (Hüttlin GmbH, Schopfheim, Germany). RA form of diclofenac in water is a clear solution manufactured by MMH using diclofenac sodium salt from Sigma or Henan Dongtai Pharm Co. as the original substance for RA forms of diclofenac-1 and diclofenac-2, respectively. Release-active dilutions were manufactured using routine methods described in the European Pharmacopoeia (7th Edition, 2011) as specified previously [12]. All ultrahigh dilutions were prepared in glass vials. The original substance (diclofenac sodium salt) was dissolved in a solvent (ethanol–water solution) and shaken for 1 min to produce the first centesimal (C1) dilution. All subsequent dilutions consisted of one part of the previous dilution and 99 parts of solvent (ethanol–water solution for intermediate dilutions and distilled water for preparation of the final dilutions). Thus, the final RA form of diclofenac in water contained release-active dilutions of diclofenac which was diluted to degrees to obtain a mixture of final C12 + C30 + C200 dilutions. Solutions were prepared in sterile conditions avoiding direct intense light and stored at RT. The quality of RA form of diclofenac was confirmed by quality control tests of the diclofenac substance in accordance with the requirements of the European Pharmacopoeia monograph 'Diclofenac sodium' (01/2008:1002), quality control of incoming materials and excipients, as well as usage of validated manufacturing process. In the case of placebo, solvent (PBS, Sigma, Cat No P4417-100TAB) was used to prepare ultrahigh dilutions instead of diclofenac powder using the same method as described above. RA forms of diclofenac and placebo were coded by the manufacturer and used blinded in the study.

Table 1
The following samples were provided by Materia Medica Holding.

Sample	State of sample	Diclofenac origin
Diclofenac-1	Powder	Sigma-Aldrich Co.
Diclofenac-2	Powder	Henan Dongtai Pharm Co.
RA form of diclofenac-1	RA form in lactose	Sigma-Aldrich Co.
RA form of diclofenac-2	RA form in lactose	Henan Dongtai Pharm Co.
Placebo	RA form in lactose	Not containing any diclofenac

2.2. Sample preparation

As diclofenac antibody source, 3 mL of diclofenac antiserum (dilution 1:7000, v/v in PBS) and 6 mL of each experimental sample (RA form of diclofenac-1, RA form of diclofenac-2 and placebo (Note: These lactose samples were used after dissolving in purified water at a concentration of 50 mg mL^{-1})) were mixed in separate eco-friendly centrifuge tubes made of polypropylene, without vertical lip (AN76.1; Carl Roth GmbH, Karlsruhe, Germany) and incubated for 60 min at 37 °C in the refrigerated benchtop shaker/incubator with orbital shaking at 700 rpm. As reference, a mixture of 3 mL of the diclofenac antiserum dilution and 6 mL of ultrapure water were incubated accordingly. Pre-incubated samples were used in ELISA.

2.3. Indirect competitive microplate-based ELISA

The microplates were washed automatically with a 96-channel plate washer (ELx405 Select), and the absorbance was measured with a microtiter plate reader (Synergy HT), both from BioTek (Bad Friedrichshall, Germany). For the indirect competitive ELISA, 200 μL per well of a 1:100 dilution in coating buffer of a diclofenac-OVA conjugate (0.1 mg mL^{-1}) were transferred to the plate which was kept overnight in a refrigerator at 4 °C (Note: plates were sealed with sealing film at this incubation point and at all subsequent points mentioned below). The coating buffer (pH 9.6) consisted of 1.59 g disodium carbonate, 2.93 g sodium hydrogen carbonate and 0.2 g sodium azide in 1 L of water. After a threefold washing step with washing buffer (400 μL/well), the plates were incubated with 1% casein in PBS (300 μL/well) under shaking at 100 rpm on an orbital flatbed shaker (Easy shaker EAS 2/4, SLT, Crailsheim, Germany) for 1 h at room temperature (RT) to block unoccupied binding sites and immediately used. For PBS, 1.36 g potassium dihydrogen phosphate, 12.2 g dipotassium hydrogen phosphate and 8.5 g sodium chloride in 1 L of water were used. The washing buffer consisted of 42 mL washing buffer concentrate (8.17 g potassium dihydrogen phosphate, 73.16 g dipotassium hydrogen phosphate, 52.6 g sodium chloride, and 30 mL of Tween 20 in 1 L of water) in 2.5 L of water. Plates were then washed again. A volume of 100 μL of diclofenac standards (Note: the number of different standard samples and used concentration range varied among experiments; in total, a concentration range from 0.01 ng mL^{-1} to 1 ng mL^{-1}, prepared with ultrapure water, was covered) and 100 μL of