

Pharmacokinetics of Der p 2 Allergen and Derived Monomeric Allergoid in Allergic Volunteers

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Key Words

Der p 2 · Monomeric allergoid · Pharmacokinetics · Radiolabelling

Abstract

Background: Presently, sublingual immunotherapy is widely used as an alternative to the injection route for respiratory allergy, but its pharmacokinetics in humans is poorly known, and data are available only for Par j 1 allergen. We aimed at assessing the biodistribution of iodine-123-radiolabelled Der p 2 in allergic volunteers. **Methods:** Purified Der p 2 and its monomeric allergoid were radiolabelled with iodine-123 and administered sublingually to 7 allergic volunteers. The subjects were allowed to swallow 6 min after administration. Dynamic (up to 10 min) and static scintigraphic images (30 min, 1, 2, 3 and 20 h) were recorded, and blood samples were obtained at different time points to measure the plasma radioactivity and to assess the presence of circulating radiolabelled species by gel chromatography. **Results:** The local pharmacokinetics did not differ between allergen and allergoid. Plasma radioactivity began to increase only after swallowing and peaked at 1–2 h. Both the allergen and the allergoid persisted in the mouth for several hours, and traces could be detectable up to 20 h. At radioactivity plasma peak, gel chromatography showed

that a fraction of the allergoid, but not the allergen, was absorbed as an intact molecule. **Conclusions:** These results indicate that the pharmacokinetics of sublingual administration is independent of the allergen used and characterized by the long persistence in the mouth. The contribution of enteric absorption of the allergoid in the mechanism of action of sublingual immunotherapy remains to be defined.

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Introduction

Sublingual immunotherapy (SLIT) was proposed for the first time in 1986 [1]. Its main rationale was to minimize or to abolish the risks of adverse events and to provide patients with a more comfortable and acceptable form of immunotherapy (IT). Initially, the sublingual administration was thought to allow a good and prompt absorption of the allergen, as with other drugs such as nitroglycerine or nifedipine, although the possibility of a too rapid absorption of the extract was regarded as a risk factor for adverse events [2]. Many clinical trials performed in Europe in the last 10 years [for a review, see ref. 3] and some post-marketing surveys [4–6] have suggested that SLIT is clinically effective and has a satisfactory safety profile; in fact, severe adverse events and life-

threatening side effects were never reported. The recent official documents [7, 8] suggest that SLIT can be a viable alternative to injection IT. SLIT was also shown capable of modulating local allergic inflammation [9, 10] and of modifying the Th2 cytokine profile [11]. Another interesting observation was that the oral route (allergen immediately swallowed) had negligible clinical efficacy, quite inferior to the SLIT. Nevertheless, the reasons for the difference were not immediately clear.

In this regard, the pharmacokinetics (absorption, degradation and fate) of allergenic extracts may contribute to explain the aforementioned clinical observations. The sparse data (mostly obtained in animal models) available on this topic often provided non-conclusive results [12]. In recent years, some pharmacokinetics studies carried out in healthy [13] and allergic [14] volunteers with the allergen Par j 1 have clarified that no direct absorption of the allergen occurs through the sublingual mucosa, whereas a fraction of the protein persists in the mouth for some hours after administration. These observations may account for the safety profile and for the clinical difference between the oral and sublingual route. At this point, it is not clear if the observed pharmacokinetics is a distinctive feature of the Par j 1 allergen or if they are common to other proteins used for IT. Therefore, we undertook the present study in order to assess the kinetics of the mite allergen Der p 2 and its monomeric carbamylated allergoid in allergic volunteers. The purified proteins were radiolabelled with iodine-123 (^{123}I), and their pharmacokinetics were studied using an established experimental protocol, involving scintiscan imaging and plasma analysis.

Methods

Study Design

We investigated the absorption and fate of the ^{123}I -radiolabelled Der p 2 native allergen and its allergoid (^{123}I -Der p 2), administered sublingually to 7 mite-allergic volunteers. The biodistribution of the radiolabelled allergen in the mouth after administration were evaluated by early dynamic and late static scintigraphic imaging. Absorption of the tracer was studied by measuring plasma radioactivity at different time intervals. Gel chromatograms at radioactivity plasma peak were also performed on Sephadex G-25 columns to check molecular mass corresponding to radioactivity.

Subjects

Seven allergic volunteers (3 men) aged between 25 and 39 years were enrolled in the study. All subjects suffered from mild to moderate/severe persistent [8] rhinitis and were sensitized to mites, as confirmed by prick test (class + + or higher) and RAST (class >1). None of them had a positive clinical history for asthma, their pulmonary function test was normal and they were free of nasal symptoms at the time of experiments. Routine laboratory parameters

(including free thyroid hormones and thyrotropin plasma levels) resulted within the normal range for all subjects. Thyroidal-free radioiodine uptake was minimized by administering a saturated potassium iodide solution (20 drops b.i.d. in the 3 days preceding the study) and potassium perchlorate (200 mg, two capsules on the day of the study). All volunteers signed an informed consent and the protocol was approved by the inner Ethical Committee.

Radiolabelled Allergoid and Allergen

Dried mites (*Dermatophagoides pteronyssinus*; Lofarma SpA., Milan, Italy) were submitted to 5% aqueous extraction in PBS overnight at 4°C under stirring. The suspension was centrifuged at 20,000 g for 30 min at 4°C and supernatant was filtered through a 0.22- μm filter. This extract had a total protein concentration (Bio-Rad protein assay; Bio-Rad, Milan, Italy) of 4.8 mg/ml and a Der p 2 content (ELISA assay; Indoor Biotechnology Ltd, Cardiff, UK) of 0.2 mg/ml. Der p 2 was further purified by affinity chromatography. Briefly, Mab DpX, a monoclonal specific to Der p 2 allergen (provided by Dr. Chapman, Indoor Biotechnologies Ltd) was coupled to CNBr-activated Sepharose (Amersham Biotech, Milan, Italy); the previously obtained extract was then applied on the affinity column equilibrated with PBS. After extensive washings, bound protein was eluted with 0.1 M buffer glycine-HCl, pH 2.5, monitoring at 280 nm UV detector. UV adsorbing fractions, neutralized with 1 M phosphate buffer, pH 8.5, were pooled and dialyzed against 5 mM ammonium bicarbonate at pH 8. The eluate was finally lyophilized and stored.

For the study, aliquots were taken, endotoxins were removed by ultracentrifugation using the Centriplus 100 device (Millipore, Milan, Italy) and samples were then checked by the Limulus Amebocyte Lysate method (LAL test; PBI, Milan, Italy) [15]. Purity and concentration of purified Der p 2 were established by SDS-PAGE and ELISA, resulting in more than 95% and 0.375 mg/ml, respectively. The monomeric allergoid was prepared by reacting the allergen with potassium cyanate at alkaline pH in order to selectively carbamylate the ϵ -aminogroups of the lysine residues [16].

The allergen and the allergoid (about 200 μg) were radiolabelled by the iodogen method [17], using 290–350 MBq (8–9 mCi) of carrier-free ^{123}I (Amersham Health Radiopharmaceuticals, Saluggia, Vercelli, Italy) at the specific activity of 7,400 TBq/ μmol or 200 Ci/ μmol . Unreacted ^{123}I -iodide was then removed by gel filtration through Sephadex G-25 pre-packed PD-10 columns (Pharmacia, Uppsala, Sweden). The final radiolabelled proteins had a specific radioactivity of 0.8 MBq/ μg (20 $\mu\text{Ci}/\mu\text{g}$). Preliminary in vitro experiments, performed with ^{125}I , showed the stability of the radiolabelled protein to deiodination over several hours in saline, serum, saliva and gastric juice. ^{123}I was chosen because of its physical properties (main γ -emission at 159 keV and a physical half-life of 13.2 h). Each subject was exposed to a radioactivity dose about ten times lower than that for a standard chest radiograph.

Administration and Kinetics Study

The dose of radiolabelled Der p 2 or allergoid (in a volume of about 200 μl) was administered sublingually by a micropipette. The approximate radioactivity dose was 7.4–15.8 MBq (200–400 μCi). Three subjects received the allergen and 4 subjects the allergoid. The tracer was always administered within 60 min after radiolabelling.

Immediately after administration, the subject was asked to close the mouth and keep the head close to the gamma camera collimator without moving for 10 min, while dynamic scintigraphic acqui-

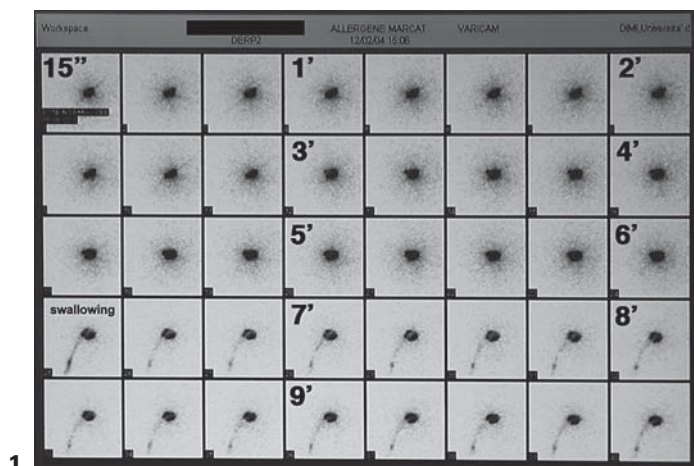
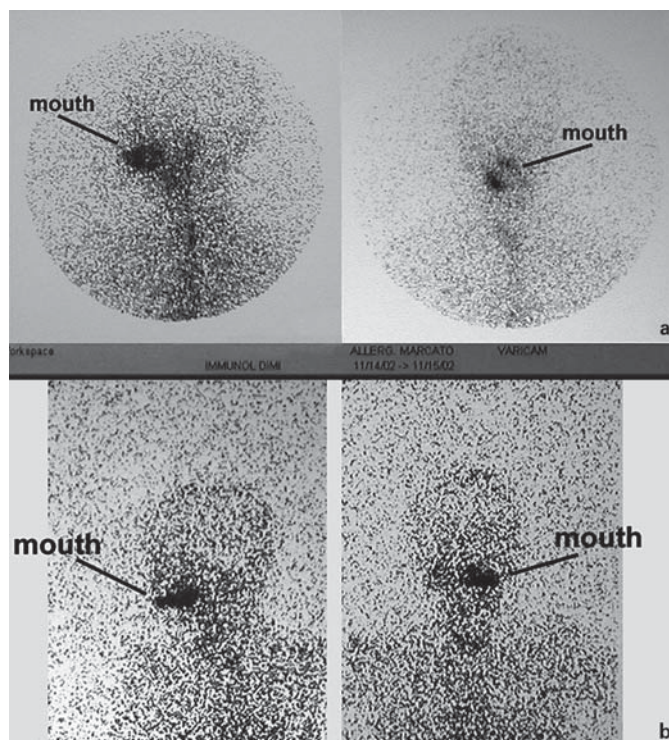


Fig. 1. Dynamic scintigraphic acquisition of the head and neck (1 frame/15 s) in 1 of the studied subjects until 10 min after administration. The mouth is oriented on the right.

Fig. 2. Static scintigraphic images (lateral view on the left, anterior view on the right) of the head and neck 3 h after the administration of the tracer. **a** Native allergen. **b** Monomeric allergoid. The persistence of radioactivity in the mouth is apparent.



sitions were performed. Swallowing was allowed 6 min after the administration. After the dynamic acquisition, the subject was allowed to move and drink. A large-field-of-view gamma camera (SP6; Elscint, Haifa, Israel) was used for scintigraphic acquisitions. Early dynamic scintigraphic acquisition upon administration of ^{123}I -Der p 2 consisted of a series of sequential images continuously recorded at the rate of 1 image/15 s, up to 10 min. After the initial dynamic imaging, single scintigraphic images (static imaging) of the head and neck were recorded at 30 min, 1, 2, 3 and 18–24 h after administration.

Heparinized 5-ml venous blood samples were collected from each subject at 0, 3, 6, 10, 30, 60 and 120 min. Plasma was separated by centrifugation and 1-ml aliquots were counted for radioactivity content in a well-type gamma counter. Furthermore, analysis of radioactive species circulating in plasma (samples obtained 1 and 2 h after administration) was performed by gel filtration with pre-packed PD-10 columns (Sephadex G-25), as previously described [13].

Results

While the labelled protein was kept under the tongue, no appreciable change was seen in the oral radioactivity, as confirmed by the selective counts performed on the appropriate 'regions of interest'. After swallowing (6 min after administration), the progression of the radioactive bolus through the oesophagus to the stomach was clearly documented (fig. 1). Also, there was a persistence of the

radiolabelled protein (either allergen or allergoid) in the mouth, and up to 3% of the administered dose was still detectable in the mouth 3 h after administration (fig. 2). Scintigraphic images at 18–20 h showed local persistence of very small amounts ($<0.1\%$ of the dose) of radioactivity as well (not shown). Immediately after administration, the local pharmacokinetics did not differ substantially between the native allergen and the allergoid (not shown).

Plasma radioactivity which was below the threshold (radioactivity background) before swallowing (3 and 6 min) became detectable at 10 min, then increased rapidly and peaked at 2 h (at 1 h in 1 subject), as shown in figure 3. Plasma radioactivity was below the threshold with the native allergen at 10 min, whereas it was detectable, although low ($3.5 \pm 1.4\%$ of plasma peak), with the allergoid. With the native allergen, plasma radioactivity appeared only at 30 min. Thus, in the present conditions, no direct absorption through sublingual mucosa could be detected. Plasma chromatographic profiles, obtained at radioactivity peak, significantly differed between the native allergen and the allergoid. With the native allergen, we could detect a large dispersion of the radioactivity (fig. 4), suggesting the presence of peptides of variable sizes, together with the free iodine. In the case of the allergoid, a small peak corresponding to the intact administered protein was detectable in all patients (fig. 5).

Fig. 3. Plasma radioactivity (normalized to plasma peak $\pm$ SEM) expressed as counts per minute (CPM) measured at different times after the administration of the allergen and allergoid. For each sample, background radioactivity was subtracted.

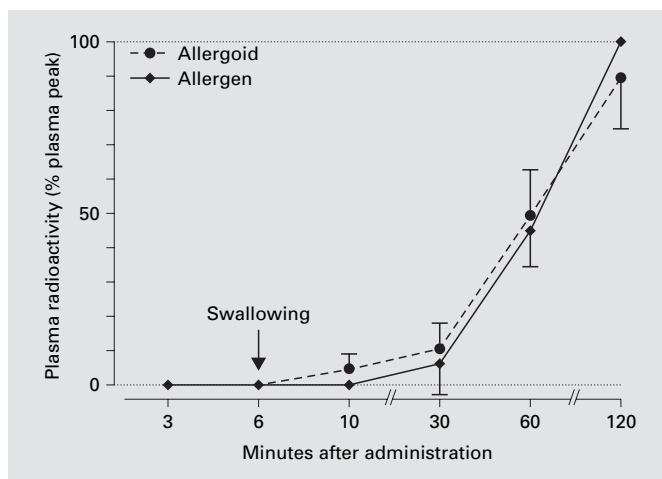


Fig. 4. Plasma chromatographic profiles (measured at plasma radioactivity peak) in the subjects receiving the ^{123}I -labelled native allergen. The labelling profile (not to scale) of the protein is plotted as dotted line for comparison.

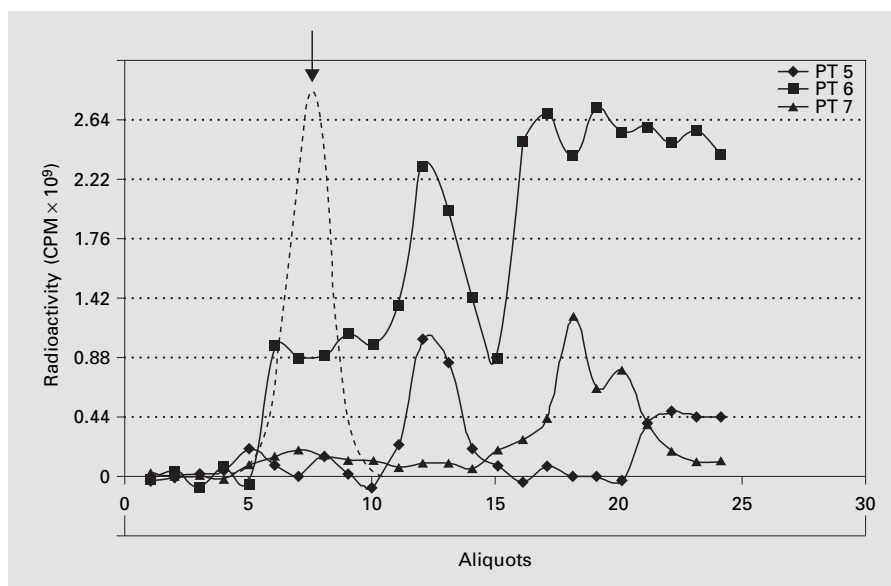
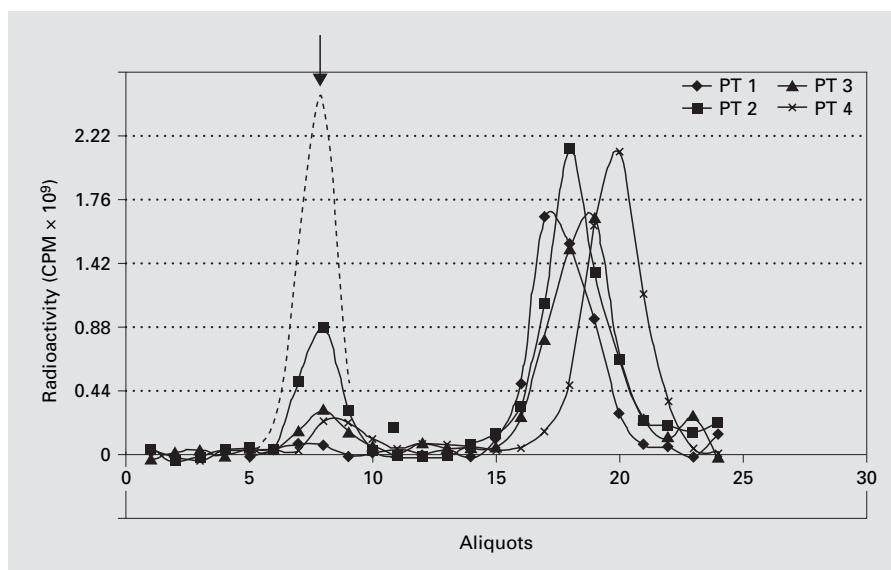


Fig. 5. Plasma chromatographic profiles (measured at plasma radioactivity peak) in the subjects receiving the ^{123}I -labelled allergoid. The labelling profile (not to scale) of the protein is plotted as dotted line for comparison.



Discussion

Although some controversies still exist, several controlled trials have shown that SLIT is clinically effective, at least in allergic rhinitis [18]. This evidence is also supported by a recent meta-analysis including 22 trials [19]. In addition, the safety profile of SLIT seems to be satisfactory in both children and adults [4–6], in contrast to the pure oral route (allergen immediately swallowed) that showed some efficacy only with very high doses of allergens, with consequent gastrointestinal side effects. This observation suggested that the contact with the mucosa is critical for the efficacy of the enteric administration. In fact, previous pharmacokinetics studies in humans [13, 14] showed that the allergen is not directly absorbed through oral mucosa, but it is retained in the mouth for hours after administration. All the aforementioned results were obtained using the purified Par j 1 allergen and its corresponding carbamylated monomeric allergoid. Thus, the following open question remained: is the observed pharmacokinetics behaviour unique to Parietaria allergen or is it reproducible with other allergenic proteins? To answer this question, we undertook a series of experiments with a different allergen. When choosing the allergen, we had to consider some aspects: molecular size, structure, clinical relevance and availability. Concerning the molecular size, some sublingual absorption could be expected with proteins smaller than Par j 1, since they could pass more easily. Nevertheless, only few allergens such as Amb t 5 and Api m 4 are quite small, but they are not good candidates. In fact, Ambrosia is not a relevant allergenic source in our country, and bee venom is not administered sublingually. The majority of relevant allergens are in the weight range of Par j 1 or much greater. Therefore, we choose the Der p 2 allergen. Its molecular weight (14.1 kDa) is not far from that of Par j 1 [20, 21], but the allergen differs in structure and function [21], is of major clinical relevance and was readily available in purified form. The allergen Der p 1 was not used because it possesses enzymatic activity and implies considerable stability problems. Since the monomeric allergoid is currently used in clinical practice [9], we decided to study its pharmacokinetics as well, in order to assess whether the chemical modifications affect the biodistribution.

As a matter of fact, the local pharmacokinetic behaviour of Der p 2 was not different from that of the Par j 1. Under the present experimental conditions, no relevant absorption of the protein through sublingual mucosa could be detected, as determined by the absence of plasma radioactivity while the allergen was kept in the mouth

without swallowing. Additionally, there was a relevant persistence of the radiolabelled protein in the mouth up to 3 h, and small traces could be detected up to 24 h after administration. Of note, the use of the sublingual-spit technique did not prove to result in substantial difference in allergen absorption [22]. As previously observed with Par j 1, the modified allergen (monomeric allergoid) seemed, to some extent, to resist gastrointestinal degradation since, unlike the native allergen, a peak corresponding to the administered labelled protein could be demonstrated in plasma by gel chromatography. The difference between the allergoid and native allergen may be due to aminogroup substitutions, which make carbamylated allergoid more resistant to digestion. Indeed, with our experimental approach, which uses a weak radio emitter, it is not possible to provide detailed information about the structural changes in molecules.

In conclusion, the biodistribution of allergens seems to be independent of the protein used and quite reproducible in humans. This further suggests that the contact with the oral mucosa is critical to the mechanisms of action of SLIT, although the pharmacokinetics studies provide only partial information on the immunological mechanism of action. The role of the protein fraction observed in the bloodstream (intact only in the case of the allergoid) in inducing the clinical effects showed in previous studies remains to be defined [9].

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