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Research Article

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A Novel Microparticulate Formulation with Allicin *In Situ* Synthesis

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Abstract

The development of Garlic as a commercial antibiotic has come to a halt. Although its efficiency is scientifically proven; it has only been used as dietary supplement or as traditional medicine. In this study we investigated the use of spray drying to obtain a powder which releases allicin, the most important antibiotic active compound in garlic. Alliin and alliinase, which serve as precursors for allicin production, were encapsulated separately into microspheres consisting of lactose as a stabiliser and as an excipient. The resulting powders, containing spray dried alliin (All_{SP}) or alliinase (Ase_{SP}), could be mixed and stored as a single entity without the risk of interaction. The particles from the combined powders dissolute the moment they come in contact with water and release alliin and alliinase which readily react to allicin (Figure 1). This production of allicin works successfully in a surfactant too. Allicin from All_{SP} and Ase_{SP} showed an antimicrobial effect against *E.coli*. Through Spray drying, particles small enough for alveolar application, can be obtained. The motive behind our formulation is the on-the-spot synthesis of the very unstable allicin. For the first time in situ synthesised allicin was made available under safe and reproducible conditions for pulmonary application. This helps treating lung infections caused by a broad spectrum of pathogens at a low level of toxicity.

Keywords

Spray drying; Microparticle; Antimicrobial; Allicin; Alliin; Alliinase; in situ synthesis

Introduction

Since ancient times *Allium sativum L.* was considered to be an important medical plant. It was described to have medicinal properties against ailments like high blood pressure, respiratory problems, indigestion and parasites. Current reports prove that most of these effects emphasise the importance of garlic as a therapeutic agent. Positive pharmacological effects on arteriosclerosis, high blood pressure, lipid metabolism, fibrinolytic activity, platelet adhesion, diabetes mellitus and neoplastic diseases were reported [1-3]. In 1944 it has been reported by Cavallito & Bailey that an extract made of fresh bulbs of *Allium sativum* (garlic) has a bacteriostatic effect against gram positive and gram negative bacteria. Their extract was an oily liquid and rather unstable. The main compound was identified as allicin, a small molecule with a thiosulfinate functional group. Following extracts or preparations containing allicin show a wide range of antibiotic activity against bacteria (e.g. methicillin-resistant *Staphylococcus aureus*, *Mycobacterium tuberculosis*), fungi (e.g. *Candida albicans*), virus (e.g. *Influenza B*), protozoa and other parasites [3-6]. In 1951 Stoll et al. discovered that allicin is a degradation product from alliin, a derivative of cysteine that can be found in high concentrations in garlic. The enzyme alliin alkyl-sulfonate-lyase (alliinase) which is also found in garlic at high concentrations, catalyses the reaction of alliin to allylsulfenic acid. Subsequently two molecules of allylsulfenic acid react spontaneously to form one allicin molecule in a condensation reaction (Figure 1). This synthesis usually occurs when the garlic bulb is damaged and is a natural defence against soil pathogens [7,8]. There are two known modes of action described for allicin and its decomposition products: antioxidant activity and protein inhibition. The first one is related to 2-propenesulfenic-acid, which is spontaneously generated at room temperature from allicin. It's trapping of peroxyl radicals could lower the rate of lipid-peroxidation, hence preventing from cardiovascular diseases. Allylsulfenic acid and 2-propensulfenic acid are synonymical terms of which the latter will be used in this article [9,10]. The second mode is its reaction with the thiol-groups in proteins. Coupling to cysteine residues causes alterations in structure and therefore in functionality. This leads to inhibition of enzymes, causing changes in metabolic redox state, protein and DNA synthesis and other enzyme dependent processes in the cell. Other proteins like tubulin have also been reported to have alterations in structure at certain allicin concentrations [3,11]. Allicin is a highly reactive/unstable molecule and forms an oily liquid, therefore pharmaceutical formulations containing allicin are difficult to produce. It is easy to synthesise but not easy to stabilise as it decomposes quickly at room temperature whether as a pure substance or as an extract in different liquids with decomposition rates in decreasing order from acetone to water to ethanol and almost complete decomposition in 1 month [12-15]. Commercial available garlic drugs are divided into 3 groups, garlic powders, garlic powder extracts and garlic fluid extracts [16]. Considerable amounts of allicin are only made available by using garlic powder which is produced by drying of garlic bulb material. This powder includes alliin and alliinase which produce allicin on contact with water. Unfortunately this garlic powder includes many other ingredients thus application is restricted to oral route [17,18]. The acid sensitivity of alliinase and the rapid degradation of allicin prevent a detectable production and systemic uptake after oral administration [19-21]. The use of spray drying to create formulations for the pulmonary application is favourable because active components are homogenous distributed and readily pulverised to particle sizes suitable for deep lung therapy. When carbohydrates are used as excipients the product will have most likely an amorphous structure which is positive for stabilisation of proteins during dehydration. The active components are protected through encapsulation and therefore decomposition is prevented. Also the encapsulation of active pharmaceutical ingredients into particulate systems helps to deliver substances that are poorly soluble or lack benefit due to fast metabolisation in the body. This might be even more interesting in the case of macromolecules like proteins because their absorption is usually blocked by different systems in the body

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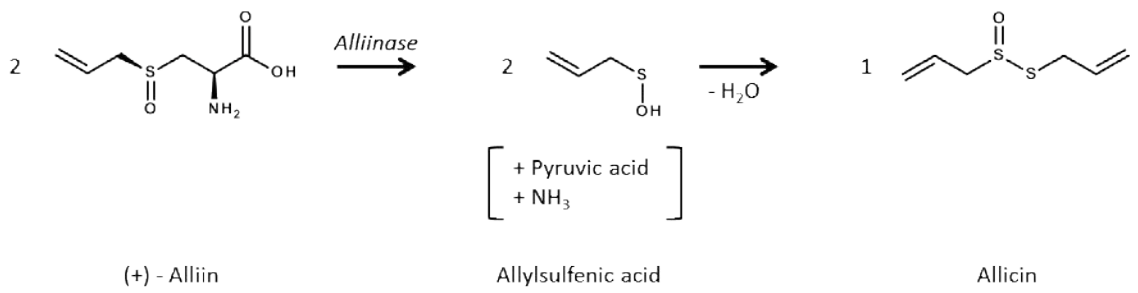

Figure 1: Reaction of combined (+) - alliin and alliinase in water. (+) – alliin is enzymatically cleaved by alliinase to allylsulfenic acid. Two molecules of allylsulfenic acid spontaneously react to give one molecule of allicin.

[22-24]. Spray drying is an established method in pharmaceutical industries which comprises easy upscaling procedure without extreme technical challenges. In case of lactose used as excipient in spray drying certain advantages come through. It is described as a safe substance for oral, inhalable or even injectable formulations. Amorphous particles from spray dried lactose dissolve very fast in aqueous conditions therefore release the active drug immediately. It is a cheap and easy available substance produced by dairy industries as a side product of cheese making. Emphasising the meaning of lactose as excipient for lung applications has been done by the fact that it is the most commonly known substance for dry powder inhaler (DPI) formulations used as a carrier for active components [25-27]. The encapsulation of active pharmaceutical ingredients into particulate systems helps to deliver substances that are poorly soluble or lack benefit due to fast metabolisation in the body. This might be even more interesting in the case of macromolecules like proteins because their absorption is usually blocked by different systems in the body. This study investigates the possibility of stabilising alliin and alliinase in lactose microspheres produced by low temperature spray drying. The resulting powders are tested for allicin production, antimicrobial effects and reasonable particle size for alveolar deposition. By focusing on in situ synthesis of allicin it addresses the major hurdles for garlic as a commercial medication in clinical use for treatment of pulmonary microbial infections.

Materials and Methods

Extraction of alliinase

Garlic was obtained from a local supermarket. The cloves were crushed and alliinase-buffer was added (7 g potassium dihydrogenphosphate, 14.5 g disodium hydrogenphosphate, 200 g saccharose, 10 g sodium chloride and 52.6 mg pyridoxal-5'-phosphate in 2l water at pH 7). The mixture was placed on ice for 15 minutes and centrifuged. Subsequently the proteins in the supernatant were then precipitated thrice using cold acetone and resuspended in Sorensen buffer (pH 7) after each step and finally resuspended in alliinase-buffer. A final centrifugation step was done and the supernatant was used for investigation. The extracted protein concentration was determined by Lowry protein assay with BSA as a standard. The amount of alliinase was determined by SDS-PAGE through measurement of the protein spots.

Synthesis of racemic alliin

The synthesis was performed according to Stoll and Seebeck [28]. About 9.7 g (0.42 mol) sodium was added to 500 mL dry ethanol, 26.6 g (0.2 mol) of L-cysteine was mixed under stirring followed by dropwise addition of allylbromide (0.22 mol). After cooling down, the solution was acidified with acetic acid to a pH of 5.5 and crystals of allyl cysteine formed. The crystals were filtrated and washed with ethanol. Oxidation was performed stepwise with 32 % hydrogen peroxide in water.

Spray drying

Spray-drying was performed using a Büchi Mini Spray Dryer B-290 (Büchi Labortechnik, Flawil, Switzerland). For spray-drying process the outlet temperature was kept constant at 50 °C and inlet temperatures were varied between 80 to 95 °C. The drying air volumetric flow rate was set at 100 L/min. The liquids for Spray drying were prepared by dissolution of the solids in double distilled water and further addition of protein extract in case of Ase_{SP} (Table 1). The liquid feed flow rate was set to 1.4 ml/min and the atomising air flow rate at 800 L/min. Compressed air from in-house supply was used to maintain drying-air and air for the atomising nozzle. The liquid feed solutions (50 mL) were atomised by using a two fluid nozzle (1.5 mm liquid orifice internal diameter). After spray-drying, the powders were collected through a high-efficiency cyclone in a glass container, transferred into glass vials and thereafter dried in a vacuum chamber at room temperature at 1 mbar for 2 days. The powders were then stored in dark in a desiccator at RT.

Particle size analysis

Size measurements of spray dried powders were done using laser diffraction analysis according to Rabbani with slight modifications [29]. The device used was a Malvern Mastersizer X with a standard flow cell and a 45 mm lens. About 10 mg of sample was dispersed in 1 mL of n-hexane (containing 0.1 % SPAN® 80) and vortexed for 10 s. The suspension was added dropwise to the flow cell filled with n-hexan/SPAN®80 to give an obscurity between 15-20 %. Calculations were done using Fraunhofer diffraction theory. The size of particles are indicated as mean volume diameter at 10 %, 50 % and 90 % of fraction, D(v,0.1); D (v,0.5) & D (v,0.9). The pulmonary deposition fraction has been calculated by subtraction of 1.04 µm fraction from 4.79 µm fraction regarding to the limits of particle diameter suitable for deposition in the deep lung [30].

Scanning electron microscopy

Small quantities of product have been mounted onto aluminium pinstubs with conductive carbon tabs/ stickers and sputter-coated 3 times with gold at 10^{-1} torr and 10 mA for one minute. Images have been acquired using a Hitachi-510 scanning electron microscope (SEM) with an acceleration voltage of 25 kV.

TLC analysis

Samples have been weighted into small cups and dissolved in 150 mM NaCl containing 2 mM Ca^{2+}. To this solution 500 µL of n-hexane was added for the extraction of newly synthesised allicin. As a positive control one garlic bulb from a local supermarket has been crushed coarsely and dispersed in 10 mL of 150 mM NaCl/ 2 mM Ca^{2+} and overlaid with 1 mL n-hexane. Both, samples and control have been incubated for 30 min at 37 °C. A suitable detection method for allicin, introduced by Keusgen [31], has been used and will be briefly described. Silica plates (ALUGRAM SIL G/UV254, 0.2 mm Kieselgel 60) were used as a stationary phase and a mixture of chloroform/methanol (99/1) as mobile phase. From each sample 10 drops of n-hexane phase was added on small spots on the plate and the solvent has been evaporated immediately. The plates were then developed. Thereafter the plates were left for drying of mobile phase and were sprayed with tin (II) chloride reagent and left for drying at 80 °C for 5 min, after this the plates were sprayed with anisaldehyde (4-methoxybenzaldehyde) reagent and developed on a heating plate (120° C) for 1-3 min until the blue spots of allicin could be seen. Images were taken immediately afterwards as the spots disappeared in about 10 minutes.

Antimicrobial activity against *E.coli*

Frozen aliquots of *E.Coli* (pTY250) were thawed on ice and diluted with 5 mL Lysogeny broth in culture tubes and then mixed in an orbital shaker for 10 minutes at 37°C. The bacterial suspension was inoculated onto agar plates (Ø 9 cm) containing LB-Miller agar. Three equidistant holes (of 5 mm each) were bored into the agar and 80 µL of sample was added into each hole. The samples analyzed were All_{SP}, Ase_{SP} (both dissolved in isotonic NaCl, c_{Alliin}=3 mg/ mL, $c_{Alliinase}$=3 mg/ mL). A mixture of equal volumes of the aforementioned samples was prepared subsequently and incubated for 5 min before adding into the holes in the agar plates (c_{Alliin}=1.5 mg/ mL). Equal volumes of Ampicillin sodium (0.75 mg/ mL) and isotonic NaCl were used as positive and negative controls respectively. All the plates were incubated overnight at 37 °C under humid conditions and checked for bacterial growth subsequently.

Alveolar modelling

About 3 mg of All_{SP} and 30 mg of Ase_{SP} have been dissolved in 200 µL of Alveofact® preparation (4 mg/ mL powder in solution of 150 mM NaCl/ 2 mM Ca^{2+}) according to Beck-Broichsitter [32]. To this solution 500 µL of n-hexane was added for the extraction of allicin and incubated at 37 °C for 30 min. After the incubation period the samples were analysed by thin layer chromatography.

Results and Discussion

Spray drying

Spray-drying promises almost perfect processing of allicin generating compounds because of the homogeneity, stability, reproducibility and scale-up possibility. The feed liquids were clear solutions in each composition due to which we obtained a homogenous mixture of its components in the feed and therefore in the resulting microparticles. The product yield of spray drying was in a medium range i.e. 44.7 % for All_{SP} and 76.9 % for Ase_{SP} (Table 2). Alliin and alliinase are described to be stable in aqueous solutions or as dry powder. Fast spray-drying of feed solutions at low temperatures supports these features. The spray drying temperature is more critical for alliinase than for alliin (gaining lower enzymatic reactivity at temperatures higher than 30 °C in water). As the outlet temperatures was around 50 °C, we assume that the decrease in enzymatic activity was insignificant (Table 2) [13,33,34]. Alliin and alliinase are enclosed in spherical lactose shells (Figure 2) which provides protection and stability. When All_{SP} and Ase_{SP} are mixed in dry state, there would probably be no interaction between alliin and alliinase. The reaction from alliin to allicin occurs only when the All_{SP}-Ase_{SP} mixture is dissolved in water. To ensure that no decomposition occurs and enzyme stays active the products need to be stored in controlled climates with low humidity, moderate temperatures and in the absence of sunlight. It is very important for formulations applied to sensitive areas of the body i.e. the lung or a dermal wound to avoid irritation caused by their ingredients. The more ingredients the more side effects could happen. Spray-drying alliin and alliinase is a simple way to produce high purity products without the typical garlic bulb material or by-products from extracts which could be found in the commercially available products [17,35,36].

Particle size

The powders Lak_{SP}, All_{SP} and Ase_{SP} comprise of spherical shaped particles, which might be due to the spray-drying process. They are hollow with a thin walled, rigid shell that contains the active compound alliin or alliinase (see Fig. 2). Mean volume diameters obtained by laser diffraction measurement indicate that the majority of particles from All_{SP} and Ase_{SP} are in the diameter range of 1 to 5 µm (Table 2). The Pulmonary deposition fractions are 74.8 % for All_{SP} and 83.3 % for Ase_{SP}. The Critical deposition size range for deep lung application in alveoli (the smallest chambers in human lung) is between 1 and 5 µm [30]. Since most of the particles in All_{SP} and Ase_{SP} fulfil this criterion, we assume a high deposition rate at this place. Only small differences in particle size could be seen between All_{SP} and Ase_{SP}. But particles in Lak_{SP} were slightly bigger which might be due to addition of alliin and alliinase which altered the drying characteristics. Furthermore the process parameters are variable allowing adaption of particle size for application to different parts of the respiratory tract.

Thin layer chromatography

To verify if a combination of All_{SP} and Ase_{SP} produces allicin

Table 1: Detailed composition for prepared formulations.

All_{SP}	150mg (+/-)-Alliin and 1350 mg Lactose anhydrous dissolved in 15ml Aqua bidest in ultrasonic bath followed by spray drying
Ase_{SP}	5000 mg lactose anhydrous dissolved in 50 ml Aqua bidest in ultrasonic-bath, addition of 5 ml Alliinase solution followed by spray drying
Lak_{SP}	5000 mg lactose anhydrous dissolved in 50 ml Aqua bidest in ultrasonic-bath followed by spray drying

Table 2: Formulation characteristics and particle size distribution.

Sample	c_{Drug} (%)	T_{OUT} (°C)	yield	D(v, 0.1) (µm)	D(v, 0.5) (µm)	D(v, 0.9) (µm)	Pulmonary deposition fraction (%)
Lak_{SP}	0	49-53	60%	1.66	4.01	7.74	43.82
Ase_{SP}	0.1 (Alliinase)	49-53	77%	1.14	2.77	4.69	83.32
All_{SP}	10 (Alliin)	50-53	45%	1.03	2.68	5.31	74.83

in a sufficient amount we used the protocol from Keusgen [31] for qualitative TLC analysis. After the reaction with anisaldehyde, following the reduction with tin (II) chloride, allicin was coloured deep blue on TLC plates (Figure 3). This colour might be due to formation of a triarylmethane dye [37]. The blue spot disappeared completely in about 10 min post staining. No blue spot could be seen from solutions of All_{SP}, Ase_{SP} and Lak_{SP} samples. Therefore no allicin has been synthesised in these samples. In our positive control, a freshly crushed garlic bulb, the blue spot was detectable at the same Rf value as in the 1:1 mixture of All_{SP} & Ase_{SP}. Although spray drying of enzymes is less usual, due to stability problems caused by several stress factors, it has been described as a method for drying while preserving enzymatic activity [38-40]. Low temperature spray drying is a safe method to stabilise alliinase and alliin which could produce allicin in aqueous solutions at detectable concentrations.

Antimicrobial effect

Allicin has a powerful inhibitory effect on many microbial pathogens, even on drug resistant strains. Its activity might be due to its rapid reaction with the thiol containing proteins and enzymes of microbes thereby interfering with their essential metabolism. Although the inhibition could be reversed by glutathione, many microbial pathogens remain sensitive because of usually low glutathione levels [41,42]. The sample solutions of All_{SP} (containing 3 mg/ mL racemic alliin) and Ase_{SP} (containing 0.2 mg/ mL alliinase) have shown no bacterial inhibition, but in contrast the combination of All_{SP} and Ase_{SP} which is thought to contain approximately 0.75 mg/ mL of allicin showed inhibitory action against *E.coli*. Similar concentrations of ampicillin do have an inhibitory effect whereas isotonic NaCl showed no action at all. In comparison with ampicillin, the All_{SP}/Ase_{SP} combination showed a smaller zone of inhibition i.e. 13.9 mm versus 25.5 mm (Figure 4). When *E.coli* has been treated with 0.01 mg/ mL Ampicillin (data not shown) the zone of inhibition is 11.8 mm and therefore a little less than the 1:1 combination of All_{SP} and Ase_{SP}. The inhibitory potency on *E.coli* of this combination of All_{SP} and Ase_{SP} is comparable to Ampicillin at the concentration of 0.01 mg/ mL. The fact that allicin is very reactive and susceptible to degradation making it difficult to determine the exact amount of allicin in the reaction mixture and hence the biologically active amount must have been even less than what we have calculated. Besides alliin existing in two different enantiomers, (+)-alliin is catalysed four times faster by alliinase and has twice the binding affinity than (-)-alliin. By using racemic alliin, this might also have been a limiting factor in the formation of allicin.

Surfactant

The field of application for this drug should be the deep lung with special interest on the pulmonary alveoli. In this part of the body the conditions to build up allicin are different than in vitro. After pulmonary application spray dried particles come in contact with different liquids like saliva, epithelial lining fluid (ELF) and surfactant. The biggest barrier for the allicin obtaining reaction might be the surfactant because of its high concentration of proteins and lipids compared to saliva and ELF [43]. This causes a higher viscosity and might increase the chance of side effects. Treating infections in the alveoli implies the complete reaction from alliin to allicin catalysed by alliinase. To test this reaction we used commercially available surfactant which simulates the conditions to be found in the alveoli epithelial lining. After 30 minutes of reaction at body temperature we were able to detect allicin by TLC-analysis with its

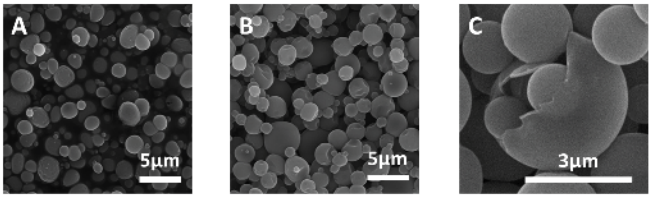

Figure 2: Scanning electron microscopic images of spray dried formulations. (A) spray dried alliin (All_{SP}); (B) spray dried alliinase (Ase_{SP}); (C) Ase_{SP} broken, hollow particle.

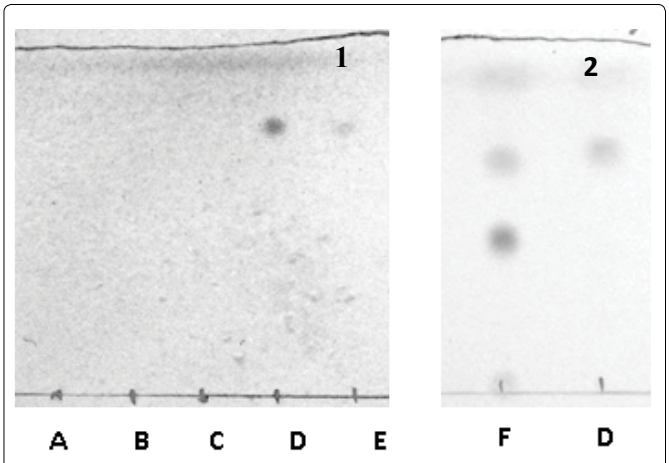

Figure 3: Quality analysis of allicin production from spray dried formulations in isotonic NaCl solution (sample A to D) or surfactant (sample F). A blue coloured spot appears if allicin is present.Plate (1) – no spot of allicin could be found in samples (A) All_{SP}, (B) Ase_{SP} and (C) Lak_{SP}. A blue spot of allicin could be found in (D) 1:1 Mix of AllSP and AseSP and (E) fresh garlic. Plate (2) – A blue spot of allicin is present in (D) 1:1 Mix of All_{SP} and Ase_{SP} in isotonic NaCl (F) 1:1 Mix of All_{SP} and Ase_{SP} in surfactant. The second spot in (F) at Rf = 0.45 was not identified.

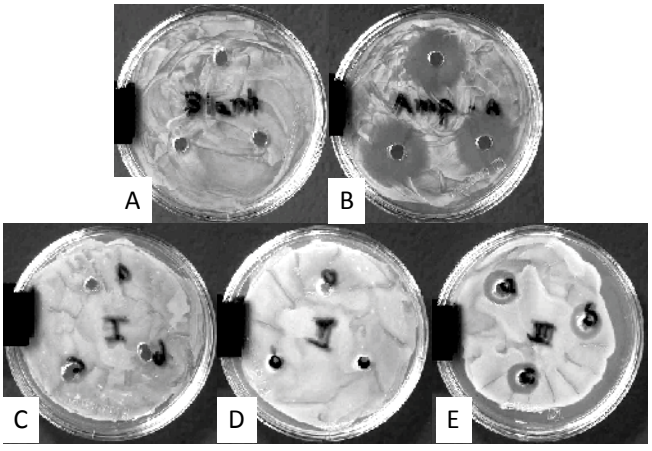

Figure 4: Photographs from LB-Miller agar cultures of *E.coli* treated with different formulations. No inhibition of growth could be seen in (A) isotonic NaCl as negative control, (C) All_{SP} in isotonic NaCl and (D) Ase_{SP} in isotonic NaCl. Whereas an inhibition of growth could be seen in(E) allicin producing 1:1 mixture of All_{SP} & Ase_{SP} in isotonic NaCl in (E) and in (B) Ampicillin Sodium in isotonic NaCl (c=0.75 mg/ml).

blue spot at usual Rf-Values (see Figure 3). The additional purple spot might be a product of a different reaction than allicin. Its identity has not been further investigated. No production of allicin occurs in Alveofact® solutions from All_{SP} or Ase_{SP} alone (data not shown). After successfully testing our drug in an alveolar simulating experiment we predict that the same reaction would occur in vivo at the site of infection.

Conclusion

The antibiotic and otherwise active ingredient of garlic, allicin, has become readily usable for the first time without any complicated stabilisation processes and can be readily produced at the area of interest, i.e. site of infection. Treating lung infections seems reasonable because particle size and reaction in a lung simulating environment give positive results. In principle it is possible to use this drug for other non-parenteral formulations. The only limitation to other applications is that no water should be used due to stability concerns. In case of infections with antibiotic resistant bacteria it could serve as a reserve antibiotic because of its different mode of reaction compared to standard antibiotics. Quantitative analysis is necessary for comparing its antimicrobial potency to established therapies. Further investigations on stability, reaction efficiency and toxicology are to be evaluated for the use of spray dried “allicin” as a therapeutic agent.

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