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Role of pharmacoscintigraphy in evaluating the presence of bioactive compounds in natural products, with particular reference to honey

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ABSTRACT

Pharmacoscintigraphy, a nuclear medicine technique based on tracing the path of a radiolabeled drug/formulation to study its kinetics and biodistribution in the body is only a recent advancement. In the present work, role of pharmacoscintigraphy in evaluating the presence of bioactive molecules in natural products has been studied. Honey, one of the most widely used natural products with well established antibacterial properties was specifically used as an example for the study. A method of radiolabeling the aqueous extract of honey with γ-emitting technetium-99m (^{99m}Tc) was standardized in the laboratory. The radiolabeled preparation was evaluated for radiochemical purity, stability and tissue distribution using tracer technique. The blood kinetics data in rabbits after intravenously administering 37MBq of ^{99m}Tc-labeled honey preparation exhibited a biphasic blood clearance pattern characterized by a fast half-life i.e., $T_{1/2(f)}$ of 25 ± 2.3 min and a slow half-life i.e., $T_{1/2(s)}$ of 16.7 ± 1.2 h. Subsequently, an animal model was prepared with localized Staphylococcus aureus (2×10^7 cells) infection in the thigh muscle of New Zealand White rabbits. The presence of bioactive antibacterial compounds in the radiolabeled preparation was confirmed by its localization at the site of bacterial lesion and its subsequent detection by nuclear medicine scintigraphy (T/NT1.92 ± 0.03 at 24 h). *In vivo* stability of the radiolabeled preparation was further confirmed in 2 healthy human volunteers by taking dynamic scintigraphy images of the GIT region under Gamma camera after oral administration of the preparation. The work suggests that components of natural/herbal products can be radiolabeled with stability similar to other radiopharmaceuticals. We propose that pharmacoscintigraphy can therefore be used as an important tool for high throughput screening of natural products for the presence of bioactive molecules, thereby providing a method for screening useful from the non-useful compounds. This will effectively result in rapid detection of promising leads for product development from the enormous potential offered by our natural product resources.

KEYWORDS

Pharmacoscintigraphy;
Natural products;
Honey;
Technetium-99m.

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1. INTRODUCTION

Establishment of a new molecule as a therapeutic or a diagnostic agent requires extensive inputs in terms of time, money and energy. A new molecular entity, either of natural or synthetic origin has to pass through a number of stages before being passed as a therapeutic/diagnostic agent[1,2]. Moreover, although a large number of molecules are scanned but only a handful of them are found to be useful. A lot of time is therefore spent on identifying these useful molecules[3]. The sub-tropical region of the world, particularly India has a rich heritage of natural/herbal products that have been used since ages to treat various diseases[4]. However, since scientific studies are few and far between, there remains a challenge of developing scientific basis for the use of natural products in healthcare. For this, clinical research in the field is highly warranted. There is a need to bioprospect and validate the natural products using modern analytical and screening techniques for discovering new chemical entities, which could offer new insights in the treatment of a large number of ailments. In recent years, systematic efforts have been made at several leading research institutes both in India and abroad to discover novel natural/herbal drugs for human ailments and validate their efficacy[5-7].

Gamma scintigraphy is a well-established nuclear medicine technique for the diagnosis of various diseases using agents that have been tagged with gamma ray emitting radioisotopes, such as technetium-99m (^{99m}Tc). Over the years many such radiopharmaceuticals have been developed in our lab and elsewhere after radiolabeling with appropriate radionuclide[8-11]. Pharmacoscintigraphy, an application of gamma scintigraphy technique in drug development and evaluation is only a recent advancement, more so in India. In this technique, a gamma-emitting radionuclide (preferably ^{99m}Tc) is tagged with the drug molecule under investigation. The radiolabeled drug molecule is then administered via the intended route of administration and the subject is scanned under a gamma camera to provide vital information regarding the pharmacokinetics of the drug molecule[12,13].

Although natural products have long been used in different parts of the world, a systematic pharmacokinetic evaluation of these potential drug candidates has not been taken up till date. As such, studies on efficacy, quality, safety and stability of natural/herbal products are essential, and our study proposes that pharmacoscintigraphy can play an important role in carrying out these tasks.

To emphasize and prove the utility of this technique, we have used honey in this particular study for the simple reason that it is one of the most widely used natural products, with known antibacterial properties[14,15]. It is well-reported that honey inhibits a broad spectrum of bacterial species[16,17]. Here we describe a method to radiolabel honey derived from honeybees (Apis mellifera) with technetium-99m, along with its pharmacological evaluation for the presence of bacterial infection specific bioactive constituents using pharmacoscinti graphy technique.

The objective of the present investigation is to ascertain the role pharmacoscintigraphy can play in high throughput screening of natural products for the presence of bioactive molecules in them, thereby providing a method for screening useful from the non-useful compounds resulting in rapid detection of promising leads for product development.

2. EXPERIMENTAL

Honey derived from honeybees (Apis mellifera) was obtained from the Department of Entomology, Punjab Agricultural University, Ludhiana, India. Stannous chloride dihydrate was procured from Sigma Chemical Co., St. Louis, MO USA. All the chemicals used in the present study were of analytical grade.

2.1. Radiolabeling

5mg (w/v) of honey was thoroughly dissolved in 1ml water for injection. To this solution, 50-500µg of stannous chloride dihydrate (1 mg/ml) (Sigma Chemical Co., St. Louis, MO USA) in 10% acetic acid was added and pH was adjusted to 7.0 with 0.1 M $NaHCO_3$. The contents were passed through 0.22µM filter (Millipore Corporation, Bedford, MA USA) into an evacuated sterile sealed vial. 1ml of sterile ^{99m}Tc-pertechnetate (70-100MBq) was added drop wise to the vial with gentle shaking. The reaction mixture was incubated at room temperature for 15 min.

2.2. Radiochemical purity

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Radiochemical purity of a radiopharmaceutical preparation is described as the percentage of the radiolabeled ligand, free ^{99m}Tc and reduced/hydrolyzed ^{99m}Tc. The radiochemical purity of ^{99m}Tc-labeled honey preparation was assessed by ascending instant thin layer chromatography (ITLC) using silica gel coated fiber glass sheets (Gelman sciences Inc., Ann Arbor, MI USA) and solvent systems, namely 100% acetone and a solvent mixture of pyridine, acetic acid and water (3:5:1.5, v/v) as mobile phase. The radioactive contaminants were identified as reduced/hydrolyzed (R/H) ^{99m}Tc and free ^{99m}Tc-pertechnetate.

2.3. Infra red (IR) spectra of the preparation

IR spectrum of the native and ^{99m}Tc-labeled honey preparation was carried out in Perkin Elmer FTIR 2000 Spectrophotometer and the peaks of the main functional groups were recorded.

2.4. *In vitro* and *in vivo* stability

The radiolabel was tested for its *in vitro* and *in vivo* stability by ascending ITLC. For *in vitro* stability in acetone and serum, 100µl of the radiolabel was mixed in triplicate with 2 ml each of 100% acetone and human serum, respectively. ITLC was carried out to assess the labeling efficiency after incubating at 37^{0}C for different time intervals. *In vivo* stability was assessed by administering 100µl of ^{99m}Tc-labeled honey preparation to New Zealand Albino rabbits through the ear vein. Blood samples were withdrawn at different time intervals and subjected to ITLC. The counts in each centimeter were measured in polystyrene test tubes by Gamma Ray Spectrometer (Electronics Corporation India Limited, ECIL).

2.5. *In vivo* quality control

2.5.1. Blood clearance and plasma protein binding studies

Blood clearance of ^{99m}Tc-labeled honey preparation was studied in rabbits. 37MBq of the radiolabel were administered to each rabbit through the ear vein and blood samples were collected at different time intervals. The radioactivity in blood was calculated as percentage of the injected dose. From the blood samples, plasma was separated by centrifugation and the proteins were precipitated by adding equal volumes of 12.5% trichloroacetic acid (TCA) and plasma. Radioactivity in the precipitate and supernatant was measured in a well-type gamma spectrometer (Electronics Corporation India Limited, ECIL).

For *in vitro* protein binding, 0.1ml of ^{99m}Tc-labeled honey preparation was mixed with 2ml of plasma. Samples were processed as above and evaluated for protein binding at different time intervals. The plasma protein binding was expressed as a fraction of total activity in the sample.

2.6. Organ distribution studies

Animal experiments were approved by the Social Justice and Empowerment Committee for the purpose of control and supervision of experiments on animals, Ministry of Government of India, New Delhi. *In vivo* distribution of ^{99m}Tc-labeled honey preparation was studied in 2-3 month old Balb/c mice. 50µl of the preparation (40KBq) was administered through the tail vein to each mice weighing 25-30g. The animals were sacrificed at different time intervals (1, 4 and 24 h post tracer administration) and different organs were removed, washed with normal saline, and dried in the paper folds. The radioactivity in each organ was counted using well-type Gamma Spectrometer (Electronics Corporation India Limited, ECIL) and expressed as percent injected dose per organ.

2.7. Bacterial animal model and scintigraphy

2.7.1. Animal model

Bacterial infectious lesion was developed in the contra-lateral thighs of healthy New Zealand White rabbits each weighing about 2kg by giving intramuscular injection of 10^7 live *S.aureus* bacteria in growing phase in 100µl volume. Swelling, redness and hyperthermia were evident in the infectious lesions after 48 h. The rabbits were kept under observation and the repeated i.m. injection of S. aureus at the same site was given after 1 week. The lesion developed within 2 weeks. The aspiration cytology of the lesions showed presence of live *S.aureus* at infection site, thereby confirming the lesion.

2.7.2. Gamma scintigraphy

2.7.2.1. In animal model

Scintigraphy in animal model was carried out after

intravenously administering ^{99m}Tc-labeled honey preparation (37MBq) in the ear vein of New Zealand white rabbits. Imaging was performed at different time intervals post administration. The rabbits were sedated with 0.75ml/Kg body weight of diazepam (Calmpose®, Ranbaxy Laboratories Limited) injection administered intravenously and 1mg/Kg body weight of Ketamine (Aneket™, Neon Laboratories) as a muscle relaxant intramuscularly 15 min before imaging under the ECIL Gamma Camera. Differential accumulation of the radiolabeled preparation in bacterial lesion was observed.

2.7.2.2. In normal human volunteers

Two healthy adult male volunteers (age 24 and 26 years) were orally administered 10mCi (370MBq) ^{99m}Tc-labeled honey preparation. Two-minute dynamic images of GIT region were taken for 30 minutes under the Gamma camera using standard imaging protocol to assess the stability of radiolabeled preparation. The digital data was used for direct visual comparison.

3. RESULTS AND DISCUSSION

The use of natural products in treatment of various diseases is a common practice in many parts of the world. However, there has been a general decline in interest in finding bioactive natural products at several major pharmaceutical companies, since the research in this field is time consuming, complex and ineffective[18]. More rational and faster strategies are required for an efficient selection of natural products containing bioactive compounds from the multitude and biodiversity of natural products that are available in the plant or the animal kingdom.

Here we suggest the use of pharmacoscintigraphy, which can play a very important role in screening of natural products by virtue of the presence of certain desirable bioactive compounds in them, thereby providing a way to facilitate and expedite the natural product research and development. Since the main constituents of most of the natural products derived from various sources such as plants, animals, insects or microorganisms are either polysachharides or polypeptides, they can provide suitable binding groups for optimum labeling with technetium-99m (^{99m}Tc).

In this particular study, honey was radiolabeled with

TABLE 1: Migration values (Rf) of ^{99m}Tc-pertechnetate, reduced/hydrolyzed (R/H) ^{99m}Tc, and ^{99m}Tc-labeled honey preparation, as determined by ascending instant thin layer chromatography with SG using two solvent systems

Solvent system	Rf value		
	Free ^{99m}Tc	R/H ^{99m}Tc	^{99m}Tc-labeled honey preparation
100% Acetone	1.0	0.0	0.0
Pyridine: acetic acid: water (3:5:1.5, v/v)	1.0	0.0	1.0

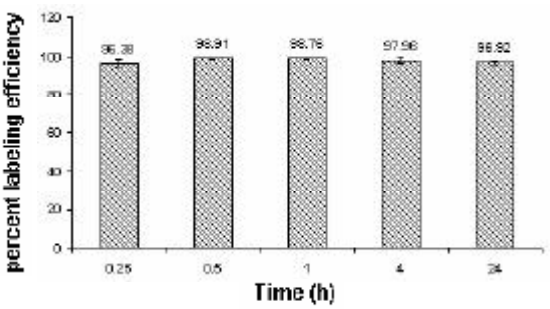

Figure 1: *In vitro* stability of ^{99m}Tc-labeled honey preparation

^{99m}Tc at pH 7.0 using 250µg stannous chloride dihydrate as a reductant. This concentration of the reducing agent at pH 7.0 yielded the highest labeling efficiency of ^{99m}Tc-labeled honey preparation. Radiochemical purity of the product was evaluated by ITLC, which successfully resolved labeled product from R/H and free ^{99m}Tc (TABLE 1). The use of two solvent systems, i.e., 100% acetone, and pyridine, acetic acid and water (3:5:1.5, v/v) was found to be a very accurate method for distinguishing and quantitating the relative amounts of free ^{99m}Tc, R/H ^{99m}Tc and ^{99m}Tc-labeled honey

Preparation

The procedure used for radiolabeling of honey was simple, which could be completed within 20-30 min. The method gave reproducible labeling efficiency.

Aqueous extract of honey showed strong bands for the presence of -COOH, -OH and -SO_3 vibration in the IR spectra suggesting strong binding groups for ^{99m}Tc. MS data suggest the presence of peptide sequence in ESI +ve mode (patent filed), which confirms the presence of organic molecules in the extract, as reported in the literature[19].

^{99m}Tc-labeled honey preparation was fairly stable *in vitro* even up to 24 h, and the percent of labeled product was 97% as determined by ITLC (figure 1).

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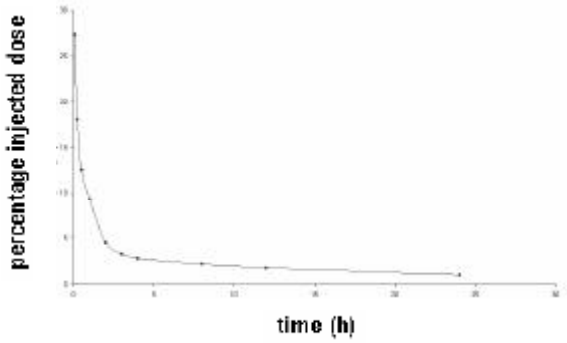

Figure 2: Blood clearance of ^{99m}Tc-labeled honey preparation in rabbit (average data of 2 rabbits)

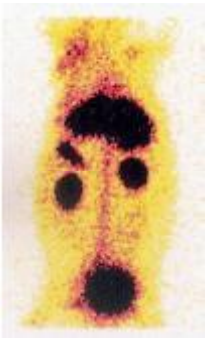

Figure 3: Whole body scintigram of a normal rabbit at 1 h after intravenous administration of ^{99m}Tc-labeled honey preparation

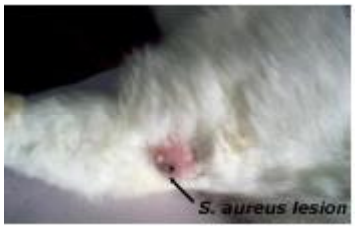

Figure 4: Animal model of a rabbit bearing a localized *S. aureus* infection on the right thigh muscle

TABLE 2: Tissue distribution in normal mice at 1, 4 and 24 h after intravenous administration of ^{99m}Tc-labeled honey preparation

Organ	Percent injected dose/whole organ		
	1 h	4 h	24 h
Blood	1.62 ± 0.18	0.79 ± 0.07	0.23±0.05
Heart	0.08 ± 0.01	0.05 ± 0.01	0.013 ± 0.005
Liver	2.19 ± 0.07	1.66 ± 0.05	0.77 ± 0.05
Lungs	0.21 ± 0.01	0.14 ± 0.01	0.07 ± 0.01
Intestine	1.87 ± 0.33	1.46 ± 0.31	0.66 ± 0.05
Kidneys	5.38 ± 0.62	3.97 ± 0.26	1.35 ± 0.14
Spleen	0.08 ± 0.02	0.07 ± 0.01	0.03 ± 0.00
Stomach	0.58 ± 0.07	0.53 ± 0.03	0.59 ± 0.12
Muscle	0.68 ± 0.17	0.17 ± 0.03	0.08 ± 0.02

Data is expressed as percent injected dose per whole organ ± SD of 5 animals

The *in vivo* stability of the complex was evident from lack of affinity of ^{99m}Tc-labeled honey preparation for stomach, which is the target organ for free ^{99m}Tc upon intravenous administration. The blood kinetics data in rabbits after intravenously administering 37MBq of ^{99m}Tc-labeled honey preparation exhibited a biphasic blood clearance pattern. The pharmacokinetic parameters were calculated by plotting percent-injected dose versus time (figure 2). The two different phases of the curve for ^{99m}Tc- labeled honey preparation suggest a fast and a slow phase of blood clearance characterized by a fast half-life ($T_{1/2(f)}$) of 25±2.3 min and a slow half-life ($T_{1/2(s)}$) of 16.7±1.2 h. The data suggests that the radiolabeled preparation has a very fast clearance from the body. *In vivo* protein binding was 66.76% at 1 h, which remained more or less constant at 24 h.

Organ distribution data of the ^{99m}Tc-labeled honey preparation in 2-3 month old Balb/c mice is shown in TABLE 2. Based on the percent-injected dose per organ, the highest uptake of ^{99m}Tc-labeled honey preparation was found in kidneys, liver, intestines and blood. This uptake pattern is similar to the one obtained for another natural product, Terminalia chebula[20], which re-enforces our claim of using radiolabeling techniques in natural product research. Scintigrams of normal New Zealand white rabbits given ^{99m}Tc-labeled honey preparation showed it to be concentrated in kidneys, liver, spleen, and urinary bladder. It cleared rapidly via the kidneys resulting in minimal of tissue background activity (figure 3). Scintigraphy of New Zealand white rabbits infected with *S.aureus* in the right thigh muscle (figure 4) showed an uptake of ^{99m}Tc labeled honey preparation at 4 h up to 24 h post administration at the site of infection, confirming the utility of pharmacoscinti graphy in establishing the presence of antibacterial bioactive molecules in honey. Region of interest (ROI) was created around the lesion which is a standard nuclear medicine practice and it was compared to the normal left thigh muscle. The target-to-non target ratio was calculated to be 1.28±0.02 at 4 h and 1.92±0.03 at 24 h, indicating that the radiolabeled honey preparation was getting accumulated at the site of infection.

Dynamic scintigraphic study in two normal volunteers subsequently gave an additional proof of the stability of radiolabeled-honey preparation (figure 5). Oral administration of the preparation in volunteers resulted

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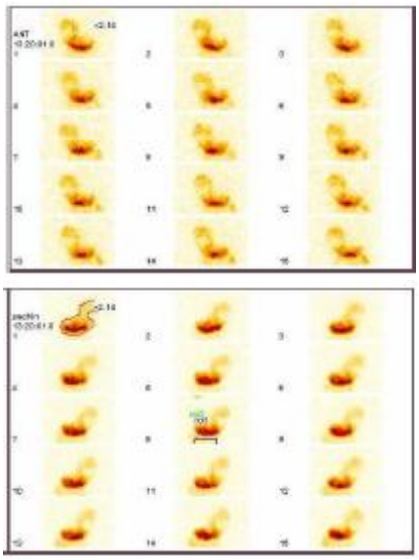

Figure 5: Dynamic scintigraphy image of the stomach in a normal volunteer after oral administration of ^{99m}Tc-labeled honey preparation

in its getting slowly absorbed from the stomach into the blood pool. No breakdown of the radiolabeled compound in acidic environment of the stomach was seen.

As already mentioned earlier, honey was chosen for this particular study since it is known to have antibacterial bioactive components[21]. Similar findings obtained with scintigraphic evaluation confirm the role of pharmacoscintigraphy and objective of this study. Natural products often serve as lead molecules whose activity can be enhanced by manipulation through combinatorial and synthetic chemistry[7]. Pharmacoscinti graphy can play a vital role in identifying these lead molecules.

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