Chemical Composition and Biological Activities of Some Extracts from *Anemone narcissiflora* subsp. narcissiflora

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ABSTRACT

The antioxidant and antimicrobial activities of essential oils, methanolic and chloroform extracts from different parts of *Anemone narcissiflora* subsp. narcissiflora from Türkiye were studied. Antioxidant activities were measured employing free radical, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$), scavenging ability of the samples. Total phenolic substance was measured for only methanol extracts. The extracts showed moderate free radical scavenging activity. Methanolic leaf extracts showed stronger inhibitory activity against $DPPH^{\cdot}$ radical with an SC_{50} value (the antioxidant concentration to achieve 50% radical scavenging) of 29 µg/mL. The antimicrobial activity was studied by the agar dilution minimal inhibitory concentration (MIC) assay and agar well diffusion assay using seven bacteria, *Escherichia coli, Klebsiella pneumoniae, Yersinia pseudotuberculosis, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus* and *Bacillus cereus*, and two yeast-like fungi, *Candida tropicalis* and *Candida tropicalis*. Methanolic and chloroform extracts were effective against the bacteria except for *B. Cereus* and the fungi. The MIC values for the extracts ranged from 12.5 to100 µg/mL, in many cases indicating better antibacterial activity as compared to the standard antibacterial ampicillin. In contrast, the essential oils from the plant showed slight antimicrobial activity only on *B. cereus* at 500 µg/mL.

KEYWORDS: *Anemone narcissiflora*, antioxidant, antimicrobial, DPPH, essential oils

Introduction

Anemone L. (Ranunculaceae) is represented with eight native species in Türkiye [1]. They are all rhizomatous and their rhizomes are exported by the local plant collectors [2]. Flowers together with the upper part of *A. coronaria* L. are used in Anatolian folk medicine against wound [3]. *A. nemorosa* L., is a poisonous plant, is used in Bulgaria. Its poisonous effect reduces after drying [4]. *A. narcissiflora* is a rhizomatous perennial herb, distributed in North-East Anatolia has two subspecies, *A. narcissiflora* subsp. *narcissiflora* and *A. narcissiflora* subsp. *willdenowii* (Boiss.) Davis [5]. Several Anemone spieces are naturally distributed in Rize, Türkiye, where the most famous and the most expensive honey type. Anemone spieces are found in Anzer plateu and are expected to be an important floral source for Anzer honey productions. Anzer honey has curative effects against many illnesses such as farangitis, tonsillitis, ulceration, heart and vascular diseases, infertility, cancer, anemia and skin care [6].

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Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important in the protection of many diseases [7, 8]. Several antioxidants compounds synthesized by plants as secondary products, especially phenolics, could play a major role in enhancing the antioxidant system, since they behave as reactive oxygen species scavengers, metal chelators and enzyme modulators [9]. Several antioxidant methods have been developed to evaluate the antioxidant capacity of the biological samples. The most widely used antioxidant methods involve the generation free radicals and their concentration is monitored as the present antioxidants scavenge them. Radical formation and the following scavenging are applied in $DPPH^{\cdot}$ measurements [7].

The aim of this study was to determine biological activities of the essential oils, methanolic and chloroform extracts of the flowers, stems and leaves of the plant to elaborate and evaluate their potential medicinal use.

Materials and Methods

Plant Material

A. narcissiflora subsp. *narcissiflora* plants at flowering stage were collected from Anzer plateu, alpine meadows, 2650 m, Rize-İkizdere Türkiye, in June 2004. The taxonomic identification of plant materials was confirmed by a senior plant taxonomist, Kamil Coskuncelebi, in the Department of Biology, Karadeniz Technical University, Trabzon, Türkiye. The plants were dried in shadow, and the leaves, flowers, and stems were separated and ground. The voucher specimen has been deposited at the Herbarium of the Department of Biology, Karadeniz Technical University, Trabzon, Türkiye.

Isolation of the Essential Oil

The essential oil of air-dried powders was obtained though hydro-distillation (15 g of powdered plant material in 1 L pure water, 3 h) by using a Clevenger-type apparatus with ice bath for cooling system. The oils were taken by dissolving in high performance liquid chromatography (HPLC)-grade n-hexane (0.5 mL) and kept at 4 °C in a sealed brown vial until tested for biological activities.

Preparation of the Methanol and Chloroform Extracts

Dried and powdered samples (25 g) were extracted successively with 250 mL of methanol and chloroform by using a Soxhlet extractor for 6 h at a temperature not exceeding the normal boiling point of the solvent. The extracts were filtered using Whatman No 1 filter paper and then evaporated to dryness at 40 °C using a rotary evaporator. The methanolic extracts were dissolved in dimethyl sulfoxide (DMSO) and the chloroform extracts were dissolved in chloroform: DMSO solvent mixture (9:1) for antimicrobial and antioxidant tests.

Antimicrobial Activity

All the test microorganisms, seven bacteria and two yeast-like fungi, were obtained from Refik Saydam Hıfzıssıhha Institute (Ankara, Türkiye) and were as follows: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Yersinia pseudotoberculosis* ATCC 911, *Pseudomonas aeruginosa* ATCC 10145, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus 702* ROMA, *Candida albicans* ATCC 60193, *Candida tropicalis* (yeast-like fungus) ATCC 13803. The essential oils and the extracts from the flower, leaf and stem samples were diluted with the solvents to prepare sample stock solutions of 1000 µg/mL.

Agar Dilution MIC Assay

Sample solutions were added to molten MHA and PDA/Tween 20 medium at 48 °C, to give concentrations ranging from 4 to 500 µg/mL [10, 11]. The antibacterial and antifungal assays were performed in Mueller-Hinton agar (MHA) (Difco, Detroit, MI) at pH 7.3 containing 1% agar and buffered Yeast Nitrogen Base (Difco, Detroit, MI) at pH 7.0 with 1% agar, respectively. Plates prepared in triplicate were spot inoculated with 3 µL aliquots of culture in MHB adjusted to yield a density within McFarland 0.5 turbidity. Plates were incubated at 37 °C for 18 h and the MIC was determined as the lowest concentration of the sample resulting in no growth of the inoculum on two of three plates. The methanolic extracts were dissolved in DMSO and the chloroform extracts were dissolved in chloroform-DMSO (1:9) to prepare stock solutions. Chloroform-DMSO (1:9) and DMSO were used as control. Ampicillin (10 µg) was the standard drugs used as reference.

Agar Well Diffusion Assay

Simple susceptibility screening test using agar-well diffusion method as adapted earlier was used [12]. Each microorganism was suspended in Brain Heart Infusion (BHI) (Difco, Detroit, MI) broth and diluted to approximately 10^6 colony forming unit (CFU) per mL. They were "flood-inoculated" onto the surface of BHI agar and Sabouraund Dextrose Agar (SDA) (Difco, Detroit, MI) and then dried. For *C. albicans* and *C. tropicalis*, SDA was used. Five millimeter diameter wells were cut from the agar using a sterile cork-borer, and 100 µL of the sample solutions were delivered into the wells. The plates were incubated for 18 h at 35 °C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. Ceftazidime (10 µg) and Triflucan (5 µg) were the standard drugs for antibacterial and antifungal activities, respectively. The essential oils were dissolved in hexan which was used as solvent control.

Antioxidant activity

The $DPPH^{\cdot}$ free radical scavenging activity of all the extracts and essential oils was measured according to the well-known $DPPH^{\cdot}$ test with a slight modification [13]. Briefly, 750 µL sample of various concentrations (0.3, 0.15, 0.075, 0.0375, and 0.01875 mg/mL) was added to 750 µL 50 µM ethanolic $DPPH^{\cdot}$ solution. Following a 50 min incubation period, at room tempereature for methanolic extracts and in an ice bath for chloroform extracts and essential oil solutions, absorbance was read at 517 nm. Two different blanks, solvent blank being a mixture of hexane-ethanol (1:1) and sample-blank containing 750 µL extract and 750 µL solvent, were used. Butylated hydroxytoluene (BHT) and quercetin, both stable antioxidants, were used as synthetic reference. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. SC_{50} (µg/mL), the antioxidant concentration to achieve 50% radical scavenging, which was calculated from the curves drawn by plotting absorbance values for corresponding sample concentrations, was used to evaluate radical scavenging activities of the samples.

Results and Discussion

Antimicrobial activity

The *in vitro* antimicrobial activity of *A. narcissiflora* subsp. *narcissiflora* methanol and chloroform extracts were tested by using the agar dilution MIC assay with the microorganisms as seen in Table-1. Methanolic and chloroform extracts were found to have a good activity against all microorganisms except for *B. cereus*. Particularly, both methanolic and chloroform extracts of leaves and flowers exhibited a stronger antimicrobial activity. In general, weaker (MIC: 100 µg/mL) antimicrobial activity was observed for nonpolar solvent in fungi, but methanolic extract showed higher (MIC: 25 µg/mL) antifungal activity. On the other hand, both methanolic and chloroform extracts of all the three parts of the plant exhibited a good activity against *E. faecalis*. *Enterococcus* species cause complicated urinary tract infections, bacteremia, endocarditis, intra-abdominal and pelvic infections, wound and soft tissue infections, neonatal sepsis, and rarely meningitis. *E. faecalis* is the most common cause of enterococcal infections (85-90%) [14, 15]. In addition, methanolic and chloroform extracts inhibited *Y. pseudotuberculosis* bacterial growth, and flower chloroform extract was the most effective. *Y. pseudotuberculosis* and *P. aeruginosa* cause infections in humans. It is primarily a zoonotic infection with variable hosts, including domestic and sylvatic animals. The condition has been associated with food-borne infections [16].

The antimicrobial activities of the essential oils of *A. narcissiflora* subsp. *narcissiflora* were tested *in vitro* by using the agar-well diffusion method (Table-2). The essential oils showed slight antimicrobial activity against *B. cereus*. However, no antimicrobial activity was observed against the other six bacteria and two yeast like-fungi. Interestingly while methanolic and chloroform extracts were not effective against *B. cereus*, essential oils were effective only on this bacterium. *B. cereus* causes a toxin-mediated food poisoning. *B. cereus* can also cause local skin and wound infections, ocular infections, fulminant liver failure, and invasive diseases, including bacteremia, endocarditis, osteomyelitis, pneumonia, brain abscess, and meningitis [15].

The results can be considered as the first detailed document on the *in vitro* antioxidant and antimicrobial activity and chemical composition of *A. narcissiflora* subsp. *narcissiflora*. Particularly, the methanolic extracts of *A. narcissiflora* subsp. *narcissiflora* can be potentially useful source of natural antimicrobial agents and antioxidant principles to be used as nutraceuticals as well as in herbal medicine.

Antioxidant activity

The current study of *A. narcissiflora* subsp. *narcissiflora* was collected from Anzer plateu in Rize, Türkiye. After drying the samples, antioxidant activity was tested on essential oils, methanolic and chloroform extracts of flowers, leaves and stems according to $DPPH^{\cdot}$ radical scavenging method [13] by comparing with the known antioxidants BHT and quercetin. In addition, total polyphenolic content of methanolic extract was determined using catechine as standard.

Table No. 1

Screening of minimal inhibitory concentration (MIC) of methanol and chloroform extracts of *A. narcissiflora* subsp. *narcissiflora.*

Samples	Microorganisms and MIC values (µg/mL)								
	Ec	Kp	Yp	Pa	Ef	Sa	Bc	Ca	Ct
Chl-Leaves	50	50	50	100	25	50	-	100	100
Chl-Flowers	25	25	25	100	25	-	-	100	100
Chl-Stems	50	50	100	100	25	25	-	100	100
MeOH-Leaves	50	25	50	25	25	50	-	25	25
MeOH-Flowers	50	25	50	25	50	-	-	50	50
MeOH-Stems	50	50	50	25	12.5	25	-	25	25
Ampicillin	8	32	>128	32	32	2	2	2	2

Ec: *Escherichia coli*, Kp: *Klebsiella pneumoniae*, Yp: *Yersinia pseudotuberculosis*, Pa: *Pseudomonas aeruginosa*, Ef: *Enterococcus faecalis*, Sa: *Staphylococcus aureus* 3, Bc: *Bacillus cereus*, Ca*: Candida albicans*. Ct*: Candida tropicalis*.
(-): no activity (1 mg /ml); MeOH: Methanol extract; Chl: Chloroform extract.

Table No. 2

Screening for antimicrobial activity of the essential oils from *A. narcissiflora* subsp. *narcissiflora* (500 µg/mL).

Samples	Microorganisms and inhibition zone (mm)								
	Ec	Kp	Yp	Pa	Ef	Sa	Bc	Ca	Ct
Flowers	-	-	-	-	-	-	+	-	
Leaves	-	-	-	-	-	-	+	-	-
Stems	-	-	-	-	-	-	+	-	-
Hexane	-	-	-	-	-	-	-		-
Ceftazidime (10 µg)	+++	+++	+++	+++	+++	+++	+++	-	-
Triflucan (5 µg)	-	-	-	-	-	-	-	+++	+++

Results were interpreted in terms of the diameter of the inhibition zone: (-): < 5.5 mm; (+): 5.5-10 mm; (++): 11- 15 mm; (+++): = 16 mm. Ec: *Escherichia coli*, Kp: *Klebsiella pneumoniae*, Yp: *Yersinia pseudotuberculosis*, Pa: *Pseudomonas aeruginosa*, Ef: *Enterococcus faecalis*, Sa: *Staphylococcus aureus*, Bc: *Bacillus cereus*, *Candida albicans*. *Candida tropicalis* . Hexane was used as solvent control.

There are many different experimental methods by which the free radical scavenging activity can be estimated. One such method, by which total free radical scavenging can be estimated, is applied by determining the efficiency of antioxidants to scavenge DPPH· radicals. DPPH· is a stable free radical, and any molecule that can donate an electron or hydrogen to DPPH· can react with it and bleach the DPPH· absorption at 517 nm [17]. There is a reverse correlation between SC_{50} values and free radical scavenging activity. The results of the DPPH· radical scavenging activity and total polyphenolic contents are given in Table-3. Methanolic extracts showed higher DPPH· radical scavenging activity than essential oils and chloroform extracts. In the methanolic extracts, DPPH· radical scavenging activity was about two to ten times higher when compared to nonpolar extracts. The higher activity of leaf extract may be attributed to high phenolic contents. The total phenolic content of natural samples reflects to some extent the total antioxidant capacity of the sample and the phenolics are the main components responsible for biological activities, including antioxidant, antimicrobial, antiviral, antifungal and anticancer activities [9].

Table No. 3

DPPH· radical scavenging activity of *A. narcissiflora* subsp. *Narcissiflora* of methanolic, chloroform, and essential oils.

	DPPH· radical scavenging activity * ($SC_{50:\ \mu g/mL}$)	Polyphenols (% in dry extract)
EO-Leaves	425±32.5	-
EO-Flower	480±45.3	-
EO-Stem	649±68.7	-
MeOH-Leaves	29±6.4	21.4±6.4
MeOH-Flower	222±34.6	7.2±2.1
MeOH-Stem	422±70.2	15.2±4.3
Chl-Leaves	952±121.5	-
Chl-Flower	512±57.6	-
Chl-Stem	117±17.8	-
BHT	9.8±0.4	-
Quercetin	2.5±0.2	-

* All of the experimental results are presented as mean±SD of triplicate measurements.
- : not tested
MeOH: Methanol extract; Chl: Chloroform extract; EO: Essential oil

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HPLC method for the estimation of stavudine in bulk and pharmaceutical dosage forms

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A new reverse phase high performance liquid chromatographic (RP-HPLC) method was developed and used for the estimation of stavudine (d4T) in bulk and pharmaceutical dosage forms using RPC-18 column using an isocratic HPLC system. The mobile phase consisted of acetonitrile and 0.05M potassium dihydrogen phosphate (pH 4.2) in the ratio of 50:50 at a flow rate of 1 mL/min. Nelfinavir mesylate (NEM) (50µg/mL) was used as internal standard. The detection was carried out at 266 nm and the linearity was found to be in the range of 0.1 - 140 µg/mL. The retention times for drug and internal standard were 3.40 and 13.392 min respectively. Studies shown that about 100.18% of d4T could be recovered indicating high accuracy of proposed method. There was no intra-day and inter-day variation found in the method of analysis. The mean drug content in branded d4T tablet dosage forms was quantified and found to be between 99.84 and 100.16%. The method was found to be simple, precise, specific, sensitive, and reproducible.

KEY WORDS: HPLC, determination, stavudine, pharmaceutical.

Introduction

Stavudine (d4T) is a nucleoside reverse transcriptase inhibitor (NRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). Stavudine is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. It is 11-[5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methyl-1H-pyrimidine-2,4-dione (CAS Reg. No. 3056-17-5) classified under nucleoside reverse transcriptase inhibitors category of antiretroviral drugs [1].

It is a potent and highly selective inhibitor of human immunodeficiency virus type 1 and type 2 replication in vitro[2]. Stavudine inhibits the activity of HIV-1 reverse transcriptase (RT) both by competing with the natural substrate dGTP and by its incorporation into viral DNA. It is active against hepatitis-B virus in HIV-infected patients[3]. Some analytical methods for the estimation of stavudine were reported such as HPTLC[4], HPLC[5-7], LC-MS[8] and radioimmuno assay[9]. The previous literature shows that most of the HPLC methods developed earlier are of bio-analytical in nature where the drug is estimated from the blood samples.. The present study is aimed at developing a simple, reproducible, and sensitive reverse phase high performance liquid chromatographic (RP-HPLC) method for the estimation of d4T in bulk and pharmaceutical dosage forms using nelfinavir mesylate (NEM) as an internal standard (IS). The proposed method will be helpful to the pharmaceutical industry for regular quality control analysis of stavudine in the bulk and various dosage forms.

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Experiemental

An isocratic HPLC system (Shimadzu®) consisting of LC-10 AT liquid pump, SPD-10A UV-visible detector, a ODS-18 RP column (4.6 mm I.D. X 25 cm length), 25 µL Hamilton® injecting syringe and MS Windows based Single channel software (Class VP®). Afcoset® electronic balance was used for weighing the materials. Pure samples of stavudine and nelfinavir mesylate were obtained from Matrix Laboratories, Hyderabad, India. Acetonitrile of HPLC grade and potassium dihydrogen phosphate of AR grade were purchased from E. Merck (India) Ltd., Mumbai. Water used was triple distilled prepared by all glass distillation apparatus.

Chromatographic conditions:	The optimized chromatographic conditions were as follows:
Chromatograph	Schimadzu HPLC system
Mobile phase	Acetonitrile : 0.05M potassium dihydrogen phosphate (pH 4.2) (50:50)
Column	ODS C-18 RP column (4.6 mm I.D. X 25 cm length)
Flow rate	1mL/min
Detection	UV set at 266 nm
Injection volume	20 µL
Temperature	Ambient
Retention time	
of Drug	3.40 min
of IS	13.392 min
Run time	18 min.

Procedure: Stock solutions of d4T and NEM were prepared by dissolving accurately weighed 25 mg of d4T and NEM in 25 mL of acetonitrile : 0.05M potassium dihydrogen phosphate (50:50) to obtain 1mg/mL solutions. From these solutions 2.5 mL was pipetted out into 25 mL volumetric flask and diluted with the same solvent system to obtain 100 µg/mL solutions. Working standard solutions of d4T each containing internal standard (NEM) solution in the concentration of 50 µg/mL were prepared by taking required aliquots of d4T solutions and then diluted with the same solvent system. The standard solutions prepared above were injected five times into the column at a flow rate of 1mL/min. The ratios of AUC of drug to IS were calculated for each of the drug concentrations. The regression equation of drug concentration over the ratio of drug peak is to that of IS was obtained. The regression equation was used to estimate the amount of d4T in pharmaceutical tablet dosage forms.

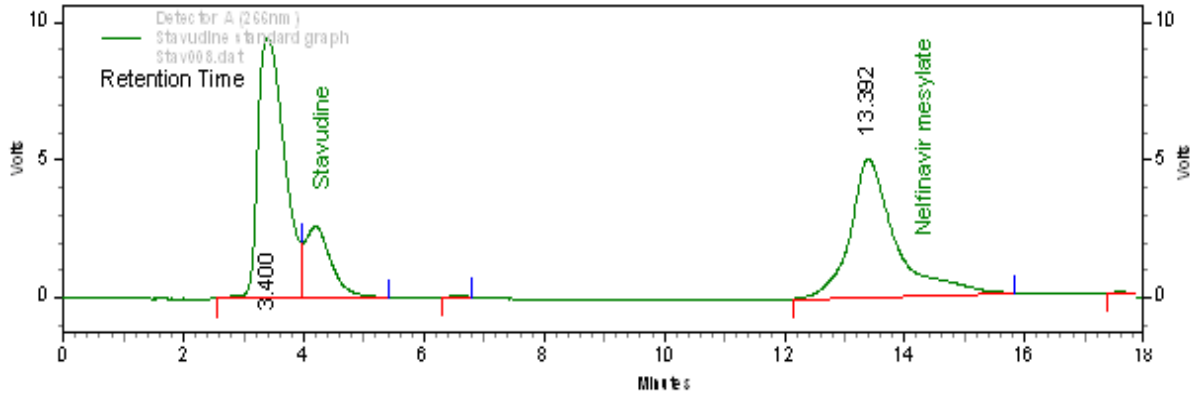
The proposed HPLC method was tested for intra-day and inter-day variations. The recovery studies were carried out by adding known amounts of (10 µg and 30 µg) of the d4T to the pre-analyzed samples and subjecting them to the proposed HPLC method.

Estimation of stavudine in its commercial tablet formulations: Contents of ten tablets containing d4T were pooled and powdered. The powder equivalent to 25 mg of d4T was extracted into acetonitrile and the volume was adjusted to 25 mL, mixed and filtered through a 0.45 µ filter. From the filtrate 0.1 mL was pipetted into a 10 mL graduated test tube and spiked with the required aliquot of IS solution and then the volume was adjusted to 10 mL with the mobile phase such that the concentration of IS in each sample was 50 µg/mL and was injected 5 times into HPLC column. The mean concentration of d4T corresponding to the ratio of AUC of d4T to that of IS was calculated from the standard graph. The same procedure was followed for remaining tablet brands.

Results and Discussion

The present study was carried out to develop a specific sensitive, precise and accurate HPLC method for the analysis of stavudine in pharmaceutical tablet dosage forms. A typical chromatogram is shown in Fig. 1. The column pressure varied from 210-220 kgf/cm^2. The retention times for d4T and IS (NEM) were 3.40 and 13.392 min respectively. Each of the samples was injected five times and almost the same retention times were observed in all the cases.

Fig. 1
Typical Chromatogram of STAVUDINE



The ratio of peak area of d4T to peak area of IS for different concentrations set up as above were calculated, and the average values for five such determinations are shown in Table-1. The peak areas of both drug and internal standard were reproducible as indicated by the low coefficient of variation (<3.18%). A good linear relationship (r = 0.9996) was observed between the concentration of drug and the respective ratio of peak areas. The calibration graph was found to be y = 0.102x + 0.0604 (where y is the ratio of peak area of drug to that of internal standard and x is the concentration of drug in the range of 0.1 to 140 µg/mL). When d4T solutions containing 10µg/mL and 30µg/mL were analyzed by the proposed HPLC method for finding out intra-day and inter-day variation, a low coefficient of variation was observed (<2.42%) showing that the method is highly precise (Table-2). About 100.18% of d4T could be recovered from the preanalyzed samples indicating high accuracy of proposed method as shown in Table-3.

Table-1

Calibration of HPLC method for estimation of STAVUDINE

Concentration of Stavudine (µg/mL)	Mean ratio of AUC of drug to IS (n=5)	CV (%)
0.1	0.01067	2.54
0.5	0.0745	2.11
1	0.1234	1.37
2	0.2503	2.15
4	0.4867	1.84
10	1.1147	1.57
20	2.0699	1.81
40	4.3378	2.16
80	8.084	3.07
100	10.39	3.18
140	14.258	1.88

C.V.= coefficient of variation, Regression equation (from 0.1 to 140 µg/ml)

Table-2

Precision of the Proposed HPLC Method

Stavudine concentration	Concentration of stavudine (µg/ml) found on			
(µg/mL)	Intra-day		Inter-day	
	Mean (n=5)	% CV	Mean (n=5)	% CV
10	10.09	1.28	10.22	1.49
30	30.15	1.84	30.31	2.42

Table-3

Recovery studies of STAVUDINE

Amount of drug added (µg)	Mean (±s.d.) amount (µg) found (n=5)	Mean % recovery
10	10.058 (±0.041)	100.13
30	30.094 (±0.066)	100.18

Table - 4

Assay of different Brands of STAVUDINE tablets

Brand	Labeled amount of drug (mg)	Mean % of labeled amount (n=5)	%CV
A	30	99.84	1.88
B	30	100.16	2.18
C	30	100.09	1.91

The d4T content in branded tablet formulations was quantified using the proposed analytical method and details are shown in Table-4. The absence of additional peaks indicated no interference of the excipients used in the tablets. The tablets were found to contain 99.84 to 100.16% of the labeled amount. The low percent of CV (<2.18 %) indicates the reproducibility of the assay of d4T in the tablet dosage forms. The proposed method was found to be simple, precise, accurate, specific and economical. Hence this method can be employed to estimate d4T in bulk and tablet dosage forms effectively.

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Formulation and *In Vitro* Evaluation of Floating Matrix Tablets of Domperidone: Influence of Combination of Hydrophilic and Hydrophobic Matrix Formers

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ABSTRACT

To develop and optimize floating tablets of domperidone, using hydrophobic and hydrophilic matrix formers (Gelucire® 50/02, and HPMC K100M respectively). Floating tablets were prepared by the melt granulation technique and evaluated for *in vitro* floating and drug release. A 3^2 full factorial design was used for optimization by taking the amounts of Gelucire® 50/02 (X_1) and Hydroxy propylmethycellulose K100M (X_2) as independent variables, and floating lag time, time required for 50% (T_{50}) and 80 (T_{80}), and diffusion coefficient as dependent variables. The results revealed that the moderate amount of Gelucire 50/02 and HPMC K100M provides desired release of domperidone from a floating system. A theoretical dissolution profile was generated using pharmacokinetic parameters of domperidone. The similarity factor f2 was applied between the factorial design batches and the theoretical dissolution profile. No significant difference was observed between the desired release profile and batches F4, F5, F7, F8 and F9. Batch F5 showed the highest f2 (f2 = 71.41) among all the batches. These studies indicate that the proper balance between a matrixing agent (HPMC K100M) and a release rate retardant (Gelucire 40/02) can produce a drug dissolution profile similar to a theoretical dissolution profile. From the present investigation it may be concluded that the hydrophobic lipid Gelucire 50/02 is an effective carrier for the design of a floating drug delivery system of domperidone.

KEY WORDS: Domperidone, Gelucire®, HPMC K100M, Floating drug delivery

Introduction

Using current release technology, oral delivery for 24 h is possible for many drugs; however, the substance must be absorbed well throughout the whole gastrointestinal tract. A significant obstacle may arise if there is a narrow window for drug absorption in the gastrointestinal tract, if a stability problem exists in gastrointestinal fluids, or the drug is poorly soluble in the intestine or acts locally in the stomach. Thus, the real issue in the development of oral controlled release dosage forms is not just to prolong the delivery of the drugs for more than 12 h, but to prolong the presence of the dosage forms in the stomach or somewhere in the upper small intestine until all the drug is released for the desired period of time [1, 2]. To overcome these limitations, several controlled oral drug delivery systems with prolonged gastric residence times have been reported recently such as: floating drug dosage systems (FDDS) [3-7], swelling or expanding systems [8], mucoadhesive systems [9, 10], modified-shape systems [11], high-density systems[12], and other delayed gastric emptying devices. Among these systems, FDDS have been most commonly used. FDDS have a lower density than gastric fluids and thus remain buoyant in the stomach without affecting the gastric emptying rate for a prolonged period of time. While the system is floating in the gastric content, the drug is released slowly from the system at a desired rate.

Gelucire are a family of vehicles derived from mixtures of mono-,di-, and triglycerides with polyethylene glycol (PEG) esters of fatty acids. Gelucire are available with a range of properties depending on their hydrophilic lipophilic balance (HLB 1-18) and melting point (33°C-65°C) range.[13,14] Gelucire containing only PEG esters (Gelucire 55/18) are generally used in the preparation of fast-release formulations, while Gelucires containing only glycerides or a mixture of glycerides and PEG esters (Gelucire 54/02, 50/13, 43/01) are used in the preparation of sustained-release formulations.[15, 16] Sutananta et al reported on sustained-release single-unit matrices using Gelucire 43/01 where only 1.7% theophylline was released over 20 hours.[17] Recently, Shimpi et al[18] reported on a multiunit floating-dosage form of diltiazem HCl, considering the benefits of a multiunit floating dosage form over other systems.

Domperidone is a synthetic benzimidazole compound that acts as a dopamine D2 receptor antagonist. Its

localization outside the blood-brain barrier and antiemetic properties has made it a useful adjunct in therapy for Parkinson's disease. There has been renewed interest in antidopaminergic prokinetic agents since the withdrawal of cisapride, a 5-HT4 agonist, from the market. Domperidone is also used as a prokinetic agent for treatment of upper gastrointestinal motility disorders [19, 20]. It continues to be an attractive alternative to metoclopramide because it has fewer neurological side effects. Patients receiving domperidone or other prokinetic agents for diabetic gastropathy or gastroparesis should also be managing diet, lifestyle, and other medications to optimize gastric motility [21]. It is rapidly absorbed from the stomach and the upper part of the gastrointestinal tract [22], after oral administration, and few side effects have been reported [19, 20]. It is a weak base with good solubility in acidic pH but in alkaline pH solubility is significantly reduced. Oral controlled release dosage forms containing drug, which is a weak base, are exposed to environments of increasing pH and poorly soluble freebase may be precipitated within the formulation in the intestinal fluid. Precipitated drug is no longer capable of being released from formulation [23, 24]. The short biological half-life of drug (7 hours) also favors development of a sustained release formulation.

Thus, the major objective of the present study was to design floating sustained-release tablets with a low drug: polymers ratio. To achieve a lower drug: polymers ratio and good floating ability, the hydrophobic grade of the lipid excipient Gelucire and Hydroxy propylmethycellulose was selected, and the formulation was optimized using a 3^2 full factorial design.

Materials and Methods

Materials

Domperidone was obtained as gift sample (Mann Pharmaceutical Ltd., Mehsana, India), Gelucire® 50/02 (waxy solid, melting point 50°C, HLB = 02) was a gift from Gattefosse (St Priest, Cedex, France). Hydroxy propylmethylcellulose K100M (HPMC K100M), Sodium bicarbonate, Ethyl cellulose (EC), and sodium bicarbonate were purchased from Laser Chemicals, Ahmedabad, India. Magnesium stearate and talc were purchased from Apex Chemicals, Ahmedabad, India. All other ingredients used were of analytical grade and were used as received.

Methods

Preparation of domperidone floating tablets

Gelucire® 50/02 was melted in a porcelain dish on water bath at 60ºC, and the required quantity of domperidone was added to the molten mass. Previously prepared geometric mixture of HPMC K100M, EC and sodium bicarbonate was added to the molten domperidone-Gelucire® mixture and stirred well to mix. The mass was allowed to cool to room temperature with stirring. The cooled mixture was scrapped form porcelain dish. The mass was passed through a 40-mesh sieve, and resulting granules were sifted on a 60-mesh sieve. The granules from 40- and 60-mesh sieves were collected and mixed with talc and magnesium stearate. This blend was compressed in tablets using 8 mm flat-face round tooling on rotary tablet machine (Karnavati Engineering Pvt. Ltd, Ahmedabad, India). Compression force was adjusted to obtain tablets with hardness in rang 4 to 5 kg/cm^2. Formulations of factorial design batches are shown in table 1.

Table 1.

Formulation and Evaluation of Batches in Factorial Design*

Batch	X_1	X_2	FLT (Second)	$T_{50\%}$ (Hour)	$T_{80\%}$ (Hour)	N
F1	20	20	87.00	5.0	12.5	0.4501
F2	20	30	75	8.1	16.3	0.4590
F3	20	40	68	8.4	16.4	0.5477
F4	30	20	134	9.7	17.6	0.5865
F5	30	30	86	11.4	19.4	0.6785
F6	30	40	67	13.1	21.3	0.6739
F7	40	20	339	11.4	19.2	0.6576
F8	40	30	280	11.6	19.6	0.6036
F9	40	40	420	12.2	20.1	0.6339

*X_1 is the amount of Gelucire® 50/02 (mg), X_2 is the amount of HPMC K100M (mg). All batches contained 30 mg domperidone, 15 mg of sodium bicarbonate, 15 mg of ethyl cellulose 2% wt/wt talc, and 1% wt/wt magnesium stearate

In Vitro Buoyancy Studies

The *in vitro* buoyancy was determined by floating lag time as per the method described by Rosa et al. [25]. The tablets were placed in a 100-mL glass beaker containing simulated 0.1N Hydrochloric acid, as per USP. The time required for the tablet to rise to the surface and float was determined as floating lag time.

In Vitro Dissolution Studies [12]

The *in vitro* dissolution study of domperidone tablets was performed using USP apparatus (model TDT-06T, Electrolab, Mumbai, India) fitted with paddles (50 rpm) at 37°C ± 0.5°C using Hydrochloric acid (pH 1.2; 900 ml) as a dissolution medium. At the predetermined time interval, 5 ml samples were withdrawn, filtered through a 0.45 µm membrane filter, diluted, and assayed at 284 nm using a Shimadzu UV/vis double-beam spectrophotometer (Shimadzu, Kyoto, Japan). Cumulative percentage drug release was calculated using an equation obtained from a calibration curve. The drug release profile is shown in Figure 1.

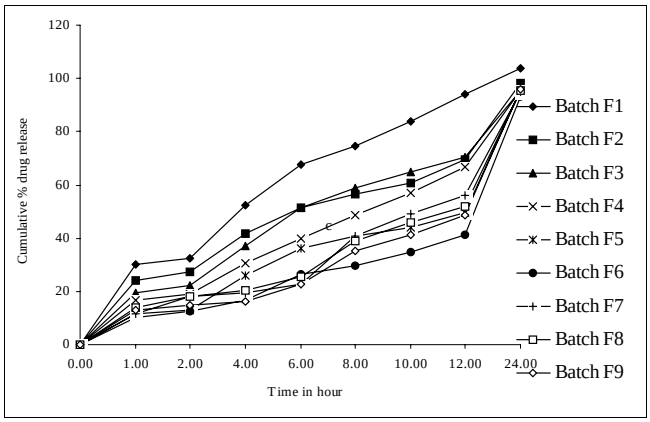

Fig.1: Release profile of factorial design batches

Optimization of Variables Using Factorial Design

A 3^2 randomized full factorial design was used in the present study. In this design 2 factors were evaluated, each at 3 levels, and experimental trials were performed for all 9 possible combinations. The amounts of lipid (Gelucire 50/02, X_1) and matrixing agent (HPMC K100M, X_2) were chosen as independent variables in the 3^2 full factorial design, while floating lag time (FLT), $T_{50\%}$, $T_{80\%}$ (i.e, time required for 50% and 80% drug release respectively) and diffusion coefficient (n) were selected as dependent variables. The formulation layout for the factorial design batches (F1-F9) is shown in Table 1, and their dissolution profiles are compared with the theoretically predicted ones in Figure 1.

Results and Discussion

Gelucire 50/02 was selected as a hydrophobic material to impart sufficient integrity to the tablets. HPMC K100M was selected as a matrixing agent, considering its widespread applicability and excellent gelling activity in sustained release formulations. Sodium bicarbonate generates CO_2 gas in the presence of hydrochloric acid present in dissolution medium. The gas generated is trapped and protected within the gel (formed by hydration of HPMC), thus decreasing the density of the tablet. As the density of the tablet falls below 1 (density of water), the tablet becomes buoyant. EC was used as floating enhancer. It also works as a dissolution retardant, being insoluble in gastric pH. Preliminary trial studies shown that floating was not achieved for tablets prepared using Gelucire. Two grade of HPMC were studied HPMC K15M and HPMC K100M as matrixing agent. It was observed that as the amount of Gelucire was increased, cumulative % drug release was decreased. Hence, it was decided to optimize the amount of Gelucire between Drug: Gelucire 1:1 ratio. As the amount of HPMC K100M was increased from drug to polymer 1:1 to 1:3 ratio, the Floating lag time increased, indicating that a high amount of HPMC is undesirable to achieve low Floating lag time. Below drug to polymer 1:1 ratio HPMC K100M might not give sufficient strength to the matrix to prolong drug release up to 12 hours. Hence, it was decided to optimize HPMC K100 M for Drug: HPMC K100M in 1:1 ratio. Fifteen mg of ethylcellulose and 10 mg of sodium bicarbonate were optimized as release rate retardant and CO_2 producing agent respectively from preliminary studies.

Calculation of immediate release part

The pharmacokinetic parameters domperidone were used to calculate a theoretical drug release profile for a 24-hour dosage form. The immediate release part for sustained release domperidone was calculated using Equation 1 and was found to be 4.211 mg.

$$\text{Immediate release part} = (Css \times Vd)/F \quad \text{.....(1)}$$

Where, *Css* is steady-state plasma concentration (Average Cmax), *Vd* is volume of distribution, and *F* is fraction bioavailable. Hence, the formulation should release 4.211 mg (14.04 %) of drug in 1 hour like conventional tablets and 1.121 mg (3.74 %) per hour up to 24 hour.

The similarity factor, f2, given by Scale Up and Pose Approval Changes (SUPAC) guidelines for modified release dosage form was used as a basis to compare dissolution profiles.[26] The dissolution profiles are considered to be similar when f2 is between 50 and 100. The method was first reported by Moore and Flanner [27]. The results of similarity factor indicate that batches F4, F5, F7, F8 and F9 full fill the above criteria. But batch F5 showed highest f2 (71.41) among all the batches. Hence batch F5 more similar compare to other batches of factorial design, similarity between theoretical dissolution profile and dissolution profile of F5 is shown in figure 2.

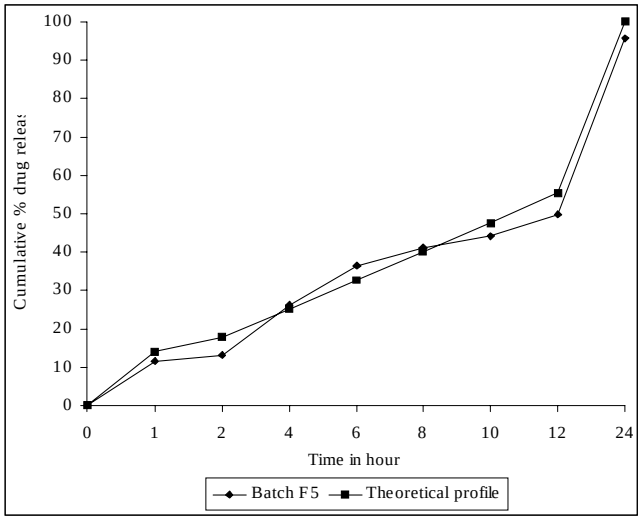

Fig.2: Comparison of *in vitro* dissolution profiles of batch F5 and theoretical dissolution profile

Optimization of variable using full factorial design

A statistical model incorporating interactive and polynomial terms was used to evaluate the responses:

$$Y = b0 + b1X1 + b2X2 + b12X1X2 + b11X12 + b22X22, \quad ...(2)$$

Where *Y* is the dependent variable, *b*0 is the arithmetic mean response of the 9 runs, and *b*i is the estimated coefficient for the factor *X*i. The main effects (*X*1 and *X*2) represent the average result of changing one factor at a time from its low to high value. The interaction terms (*X*1 *X*2) show how the response changes when 2 factors are changed simultaneously. The polynomial terms ($X1^2$ and $X2^2$) are included to investigate nonlinearity. The statistical analysis of the factorial design batches was performed by multiple linear regression analysis using Microsoft Excel. The FLT, t_{50}, t_{80}, and n values for the 9 batches (F1 to F9) showed a wide variation; the results are shown in Table 2. The data clearly indicate that the values of FLT, t_{50}, t_{80}, and n are strongly dependent on the independent variables. The fitted equations relating the response FLT, t_{50}, t_{80}, and n to the transformed factor are shown in Equation 2, Equation 3, Equation 4 and Equation 5 respectively.

$$\mathbf{FLT} = 69.778 + 134.833X_1 - 8333X_2 + 25\,X_1X_2 + 115.833\,X_1^2 + 38.83X_2^2 \quad \text{..... (3)}$$

R-square: 0.953292

$$\mathbf{T_{50\%}} = 0.638826 + 073035X_1 + 0.02694X_2 + 0.0303181X_1X_2 + 0.087636618X_1^2 + 0.011214X_z^2 \quad \text{..... (4)}$$

R-square: 0.926784

$$\mathbf{T_{80\%}} = 11.6639 + 2.2925*X_1 + 1.27147X_2 - 0.65721X_1X_2 - 1.9592171X_1^2 - 0.39599X_2^2 \quad \text{..... (5)}$$

R-squared: 0.970510218

$$\mathbf{n} = 19.8194 + 2.27898X_1 + 1.41232X_2 - 0.74347X_1X_2 - 2.0789\,X_1^2 - 0.57898X_2^2 \quad \text{..... (6)}$$

R-squared: 0.962030441

The values of the correlation coefficient indicate a good fit. The polynomial equation can be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries, (ie, positive or negative).

Table 2.
Analysis of variance table for dependent variables from factorial design

Source	SS	df	MS	F value	Prob
Floating lag time (FLT)					
Regression	141435.1111	5	28287.022	12.24586	0.0328
Residual	6929.777778	3	2309.9259		
Total	148364.8889	8			
Time required for 50 % drug release ($T_{50\%}$)					
Regression	0.055634	5	0.011127	7.594885	0.0629
Residual	0.004395	3	0.001465		
Total	0.060029	8			
Time required for 80 % drug release ($T_{80\%}$)					
Regression	50.95298	5	10.1906	19.74603	0.0167
Residual	1.54825	3	0.516083		
Total	52.50123	8			
Diffusion exponent (n)					
Regression	54.65634	5	10.93127	15.20213	0.0243
Residual	2.157185	3	0.719062		
Total	56.81353	8			

*df indicates degree of freedom; SS, sum of square; MS, mean sum of square; and F, Fischer's ratio.

Tablets of all batches (F1 to F9) had floating lag time varies from 68 second to 420 second. Polynomial equation (eq.2) for floating lag time suggests that amount of Gelucire has more significant effect on floating lag time. It may due to binding effect and hydrophobic nature of Gelucire, which prevent interaction between gas generating agent ($NaHCO_3$) and dissolution medium (0.1N HCl, pH 1.2). Figures 3 show the plot of the amount of Gelucire 50/02 (*X*1) and amount of HPMC K100M (*X*2) versus FLT. The plot was drawn using state ease (Design-Expert® version 7, Stat-Ease, Inc., Minneapolis, MN 55413). The data demonstrate that both *X*1 and *X*2 affect the floating lag time. It may also be concluded that the low level of *X*1 (amount of Gelucire 50/02) and the higher level of *X*2 (amount of HPMC K100M) favor low floating lag time. The high value of *X*1*X*2 coefficient also suggests that the interaction between *X*1 and *X*2 has a significant effect on FLT. It can be concluded that the FLT changed by appropriate selection of the *X*1 and *X*2 levels.

Time required release to 50% of drug ($t_{50\%}$) and time required release to 80% of drug ($t_{80\%}$) showed wide variation

(table 1). Figures 4 and 5 show the plot of the amount of Gelucire 50/02 (*X*1) and amount of HPMC K100M (*X*2) versus t50% and t80%, respectively. The data clearly indicate that the dependent variables ($t_{50\%}$, $t_{80\%}$) are strongly dependent on the independent variables. The fitted equation relating the response $t_{50\%}$ and $t_{80\%}$ to the transformed factors are shown in equation 3 and 4. The polynomial equation can be used to draw a conclusion after considering the magnitude of coefficient and the mathematical sign it carries (positive or negative). Data of $t_{50\%}$ and $t_{80\%}$ clearly indicate that as the amount of Gelucire increase time required to drug release increase it may due to hydrophobic nature of Gelucire which produce impermeable membrane on drug particles and prevent drug dissolution. Gelling nature of HPMC K100m also responsible sustain the drug release.

Dissolution profiles were fitted with the power law equation given by Korsmeyer and peppas [28]. Diffusion exponent range from 0.0.4501 to 0.6789 indicating anomalous drug release involving combination of swelling diffusion and/or erosion of matrices. This might be due to poor water solubility of domperidone as well as difference in characteristics of polymers. Nonlinear relationship was obtained between diffusion exponent and two independent variables. Figures 6 show the plot of the amount of Gelucire 50/02 (*X*1) and amount of HPMC K100M (*X*2) versus diffusion exponent.

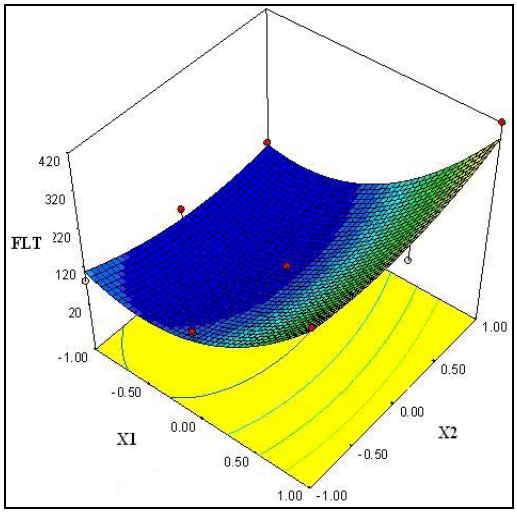

Fig.3: Response surface plot (3D) showing the effect of the amount of Gelucire 50/02 and HPMC K100M on Floating lag time.

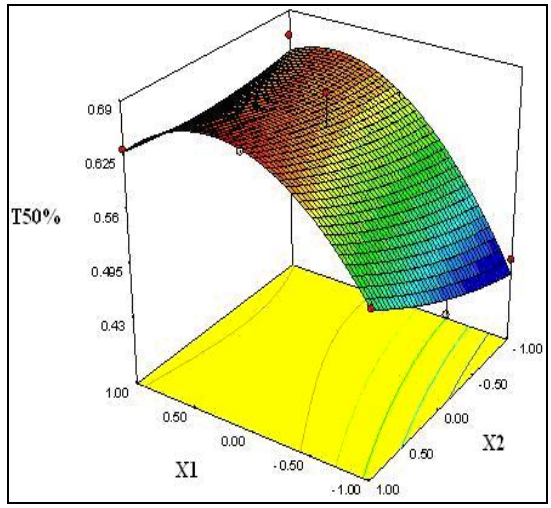

Fig.4: Response surface plot (3D) showing the effect of the amount of Gelucire 50/02 and HPMC K100M on $T_{50\%}$.

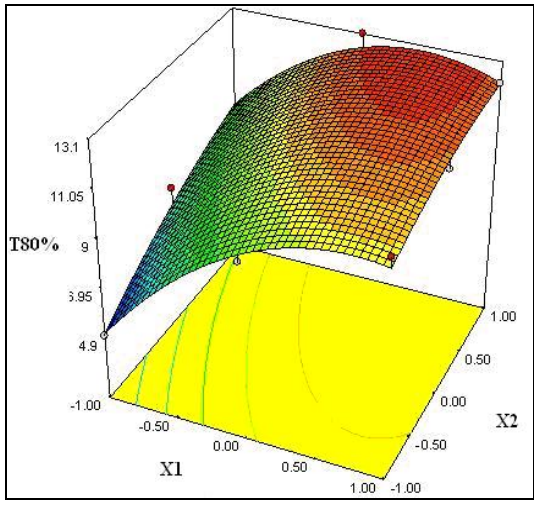

Fig.5: Response surface plot (3D) showing the effect of the amount of Gelucire 50/02 and HPMC K100M on $T_{80\%}$.

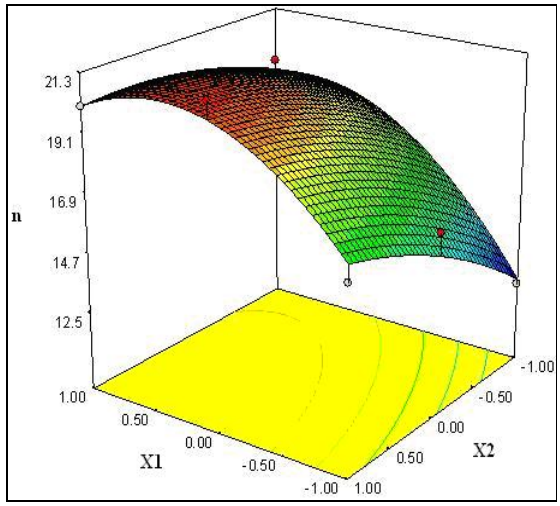

Fig.6: Response surface plot (3D) showing the effect of the amount of Gelucire 50/02 and HPMC K100M on n .

Conclusion

From the present investigation it may be concluded that the hydrophobic lipid Gelucire 50/02 is an effective carrier for the design of a floating drug delivery system of domperidone.

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Single Drop Micro Extraction and Determination of Tamoxifen as an Anticancer from Water Samples by Gas Chromatography-Mass Spectrometry

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ABSTRACT

Our investigations show that single drop micro extraction (SDME) is an efficient preconcentration method for extraction of some anticancer such as tamoxifen. A micro drop of toluene (as extracting solvent) was used in this investigation. After extraction, immediately, the microdrop was reacted back in to the syringe and injected directly in to a GC-MS injection port. The effects of nature of extracting solvent, sample temperature, ionic strength, stirring rate, microdrop and sample volume, and extraction time were verified in order to optimize the method. The linearity was studied by preconcentration of 6 ml of distillated water, spiked with a standard solution of tamoxifen at a concentration range of 0.5-5 μ*gr/ml* (RSD: 17%). the correlation coefficient and detection limits were obtained.

KEY WORDS: Single drop micro extraction, Gas chromatography-mass spectrometry, Tamoxifen, Optimization.

Introduction

Tamoxifen is antiestrogenic trogenic drug which is used in the treatment of breast cancer and nonmalignant breast disorders. It also has a stimulating effect on the secretion of hypofisor gonadotropic hormones and is generally used in treatment of infertility.

In males, tamoxifen increases endogenous production of androgenic steroids, and therefore is used by athletes [1]. Tamoxifen has been available since the early 1970, for the first-line treatment of metastasis breast cancer in postmeno pausal women and since the 1980 , it has become the therapy of choice for this condition.

The aim of our work was to find an efficient method for determination of tamoxifen in urine. Over the past decade, liquid-phase microextraction (LPME) has emerged as a novel sample preparation technique, which is simple, low cost, rapid, little sample and solvent consumption [2, 3]. This technique is based on the passive distribution of analytes between microliter volumes of organic phase and the aqueous phase. Until now, several different operational ways including static and dynamic LPME [4], hollow fiber membrane LPME [5], solvent bar microextraction [6], continuous microextraction (CFME)[7] and drop-to – drop solvent microextraction (DDSME) [8] have been developed. Single drop microextraction (SDME) is evolved from this technique in which the extraction phase is a drop of water-immiscible solvent suspended in the stirred aqueous solution. After a certain time, when sufficient amounts of analytes transferred in to the organic extractor, the microdrop is retracted in to the microsyringe, and subsequently part of all of the organic solvent is injected in to the chromatographic system. To obtain the highest possible sensitivity , the MS detection was operated using time-scheduled SIM based on the selection of three mass peaks of the highest intensity for each compound . Because of its extreme simplicity, many successful applications of SDME have been reported in many literatures [9-11]. However, the demerits of this technology such as instability of drop, relatively low precision and sensitivity [12] are often encountered.

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Materials And Method

Materials

Tamoxifen was obtained from Iran Hormon, Bromonaphtalen, methanol, toluene, xylene, butyl acetate were from Merck.

Apparatus

A 10 μ*L* SGE microsyringe was used for the extraction and injection procedures. The analyses were performed in

a Agilent technologies Hewlett packard 6890 series GC equipped with split/splitles injector and 5973 mass - selective detector system. Helium (99.999%) was employed as carrier gas and its flow rate was adjusted to 0.9 ml/min.

The separation was performed on a 30.0m× 0.25 mm i.d. fused- silica capillary column coated with a 0.25 μ*m* bonded film of HP-5 MS.

The GC column temperature was programmed at 60^0c for one min and then raised to 280^0c at 50^0c/min for 5min. The injector temperature was set at 270^0c. the GC-MS interface was maintained at 285^0c and all injections were carried out on the splitless mode. The MS was carried out on the splitless mode. The MS was operated in the total ion current (TIC) mode. For quantitative determination, the MS was operated in SIM mode. Quantitation was performed by calculating peak areas relative to internal standard.

Method

A fixed concentration of Bromonaphtalene (15mg/l) as internal standard was prepared in toluene as extracting solvent. Aqueous standard solutions of tamoxifen were freshly prepared at various concentrations in the range of 0.5 – 4 μ*g/ml*. A 6 ml of the mixture was placed in the 10 ml vial with septum. The SGE syringe was completely washed with acetone. After drying the syringe, it was rinsed and primed at least seven times with the solvent/ internal standard. On aliquot of 2-3μL of toluene containing 15 mg/lit of internal standard was withdrawn in to syringe. The syringe plunger was depressed by 0.5 μ*l* to produce a small drop at the tip of the syringe needle and then was immersed into the 6 ml stirred sample solution, that was placed in the paraffin bath for fixing temperature. The syringe was held in place by clamp. The syringe plunger was depressed to expose a 2μ*l* drop of solvent to sample solution. After extraction, immediately, the microdrop was reacted back in to the syringe and injected directly in to a GC injection port.

Results and Discussion

Optimization

The optimization approach of the tamoxifen extraction was examined by studying different parameters, including the nature of extracting solvent, ionic strength, microdrop and sample volume, stirring rate, extraction time, pH and temperature.

For extraction efficiency, the ratio of peak area of tamoxifen to internal standard (15 mg/L) was used under various conditions.

Nature of microdrop solvent

For selection of suitable solvent in order to extraction, the solubility of three solvents with different polarity and water were examined. Each of solvents containing 15ppm of bromonaphtalene as a internal standard. Extraction of stirred solution (250 rpm) was performed at 27^0c for 15 min. The extraction efficiency was based on the average peak area of analyte for three replicate analyses. The effect of nature of microdrop solvent on the analytical signal is shown in Figure1. Therefore , toluene was chosen as extracting solvent in this investigation.

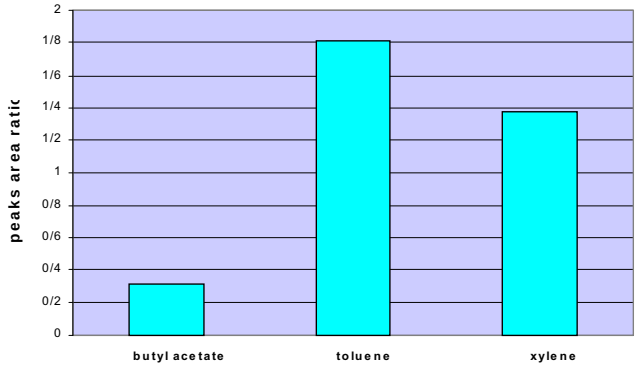

Fig.1: Effect of organic solvent on the extraction efficiency of tamoxifen from aquatic samples.

Ionic strength

A series of spiked samples with various concentrations of NaCl in the range of 0-15% were prepared by adding of calculated weight of NaCl into a 6ml volume of sample solution. Plot of relative peak area versus ionic strength has been shown in Figure 2.

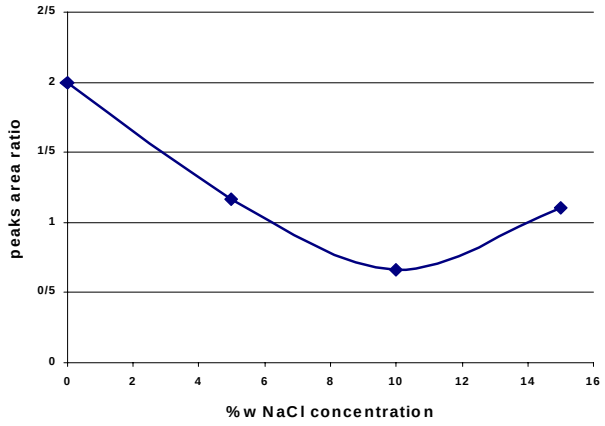

Fig.2: Effect of ionic strength on the extraction efficiency of tamoxifen

Microdrop volume

Four different volumes were investigated. The effect of microdrop volume on the analytical signal is shown in Figure 3. The curve indicate that the extraction efficiency enhances by increasing the microdrop volume from 1.5 to 2.5 μ*lit* and does not change at 3 μ*lit* compared to 2.5 μ*lit.*

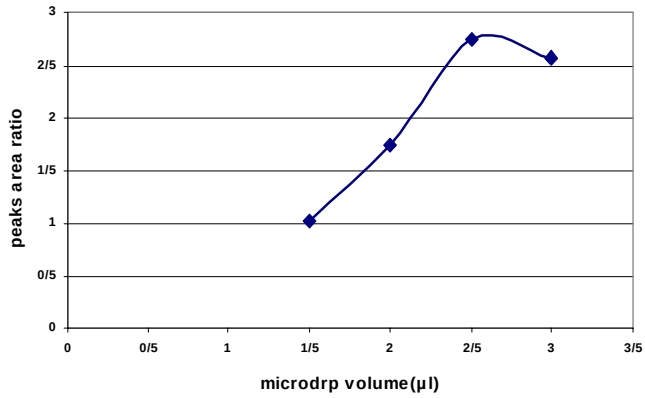

Fig.3: Effect of micro drop volume on the extraction efficiency .

Sample volume

The effect of sample volume on the extraction efficiency were examined by four samples from 4 to 7 ml. Plot of relative peak area versus sample volume were drown in Figure 4. An increase in sample volume, enhances the extracted amount of analyte in headspace SME [13,14]. But in SME by single drop, for fast applications, V_{aq} must be minimized. However, to preventing of detection problems, sample volume must be optimized. An increase in response was observed for these compounds upon increasing the sample volume up to 6 ml. But in 7 ml, a decrease in extraction was observed. This observation can be explained by this fact that by stirring the solution at a fixed rate with a longer volume, the convection is not as good in the aqueous phase, resulting less extraction.

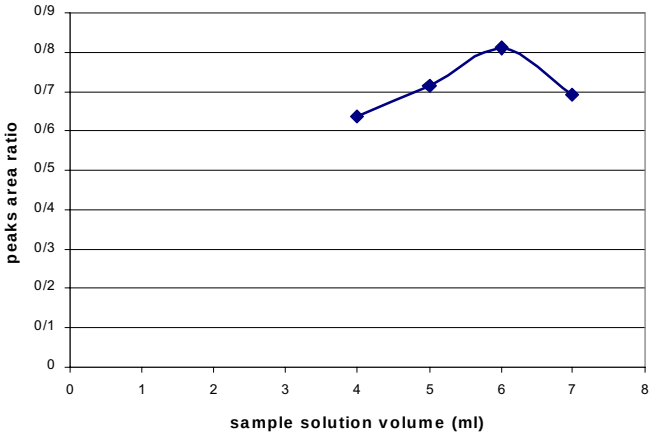

Fig.4: Effect of sample volume on the extraction efficiency.

Stirring rate

Stirring rate plays an important role in SDME analysis. Stirring the solution improves mass transfer in the aqueous phase. Therefore, equilibrium between aqueous and organic phases enhancing by increase of the stirring rates from 250 to 500 rpm.

The film theory of convection – diffusive mass transfer [15] at steady state, indicates that the mass transfer coefficient in the aqueous phase increases by increasing stirring rate because faster agitation can decrease the thickness of diffusion film in aqueous phase, higher stirring rate results in dislodgment of the drop due to the small contact area. The effect of stirring rate on the analytical signal is shown in Figure 5. Therefore, in other extractions we used 5oo rpm as optimum stirring rate.

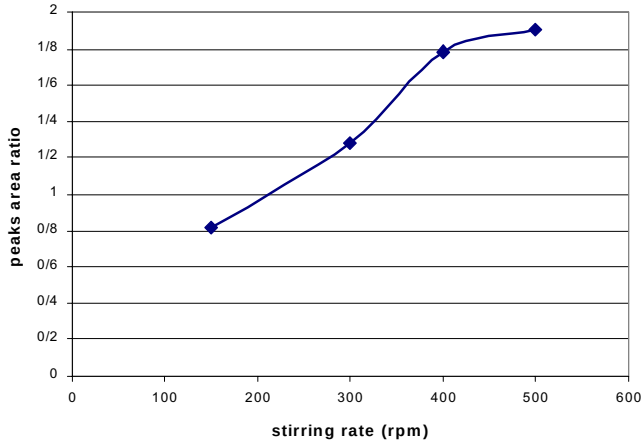

Fig.5: Effect of stirring rate on the extraction efficiency of tamoxifen

Extraction time

Extraction time enhances amount of analyte that was interested to micro drop. For analytes with high molecular weight, low concentration on SDME is expected and so longer time periods is needed to reach to equilibrium. In this investigation, the effect of extraction time was studied in the range of 10-25 min at room temperature , using 5oo rpm stirring rate. Increasing effect of extraction time are shown in Figure 6. Then we have chosen 25 min. for extraction time as optimum. Also, it is important to mention that, a long extraction time could decrease the size of microdrop.

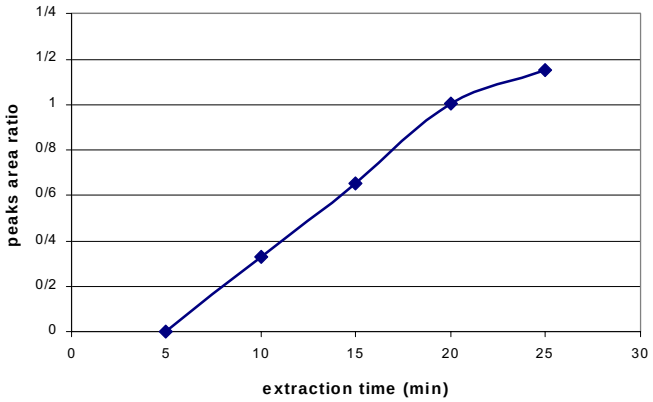

Fig.6: Effect of extraction time on the extraction efficiency.

pH effect

It is known that for a weak organic acid or base, the existence form of certain analytes will change with the change of solution pH and thereby affects on water-solubility and extractability. So prior to extraction, sample solutions are often adjusted to appropriate pH value. In this work , a range of sample pH from 8 to 12 was verified. By increasing pH, the extraction efficiency was decreased. The experimental results indicated that the highest extraction efficiency was achieved in neutral conditions (Figure 7). Thus sample solution was adjusted to neutral pH in the following experiments.

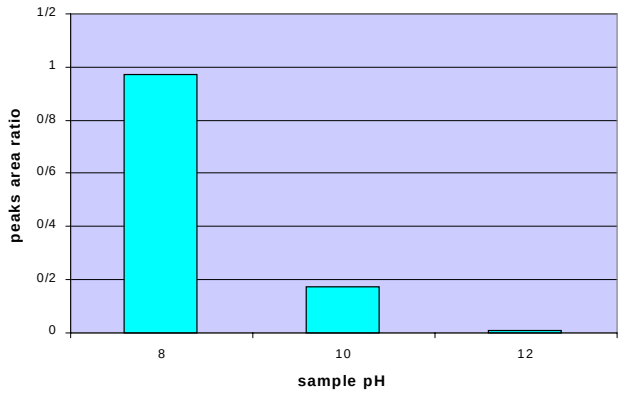

Fig.7: Effect of pH on the extraction efficiency of tamoxifen from aquatic samples

Temperature effect

The microdrop of sample solutions were exposed for 25 min., at different temperatures in the range of 27 to 55^0c. As Figure 8 demonstrates, with increasing the sample temperature, extraction efficiency is enhanced. This is expected behavior, since at elevated temperature, the mass transfer coefficients will be enhanced. But at higher temperature, irregular formations of bubbles could cause significant effects on the drop stability. Therefore in this research, 50^0c was used for extraction.

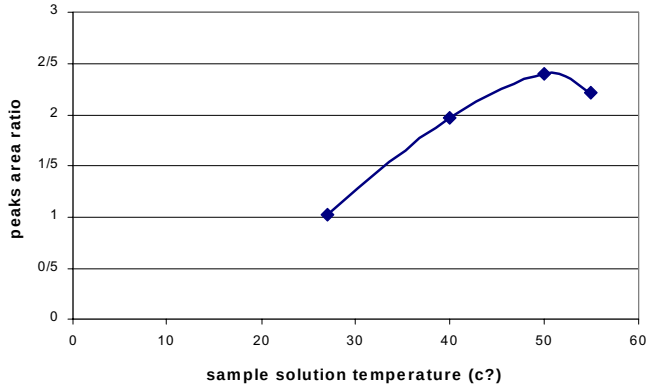

Fig.8: Effect of sample solution temperature on the extraction efficiency of tamoxifen

Evaluation of the method performance

Chromatogram of the standard solution of analyte after extraction with 2.5 µ*l* drop of toluene containing 15 ppm bromonaphthalene as internal standard is shown in Figure 9. Calibration curves were drawn using 5 spiking levels of tamoxifen in the concentration range of 0.5-5 µg ml^{-1}. For each level three replicate extractions were performed at optimal conditions.

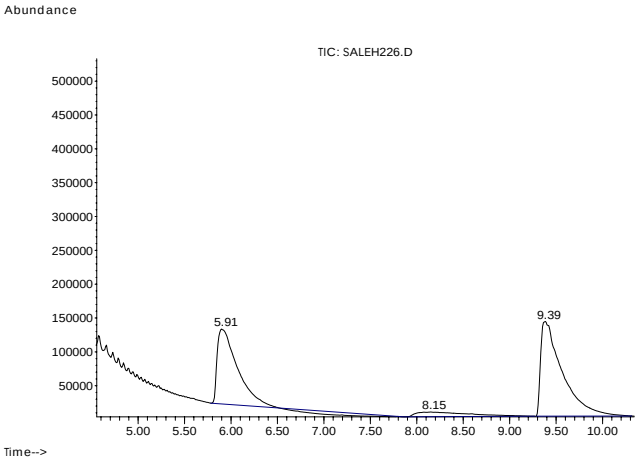

Fig 9: Chromatogram of the standard solution of analyte after extraction

The corresponding regression equation, correlation coefficient (R^2) and limit of detection (LOD) are shown in table 1 .

regression equation	correlation coefficient	LOD
Y = 0.5267x -0.2362	0.9907	0.08µg ml^{-1}

Table 1: Quantitative data obtained after SDME

The enrichment factor was calculated as the ratio of the final concentration of the analyte in the microdrop and its concentration in the original solution. The results are shown in table 2.

concentration (µl)	1	2	3
Enrichment factor (Ef)	180.27	175.48	148.12

Table 2: Enrichment factor in the standard solution

Finally, the applicability of this extraction method was investigated to real fresh urine samples of healthy male who consumes 40 mg tamoxifen every day for about two years. Figure 10 shows the mass spectrogram obtained after immersed SME urine real sample.

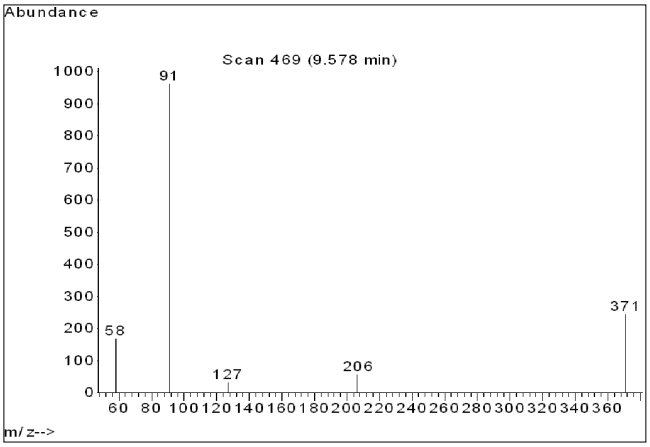

Fig.10: Mass spectrum of real sample. conditions :microdrop volume : 2.5µl, pH : 8, stirring rate :500rpm ,extraction time :25 min, sample volume :6ml , temperature :50ºc ,without salt addition.

SME is a non-exhustive extraction procedure like SPME and for this reason, the relative recovery difined as the ratio of GC-MS peak areas of spiked urine extract was used to spiked bedistillated water extract. Figure 11 (a,b) shows two mass spectrograms obtained after immersed SME of 6 ml urine sample spiked with 5 μg.ml^{-1} tamoxifen (a) and 6 ml of bidistillated water spiked with 5 μg.ml^{-1} tamoxifen (b).

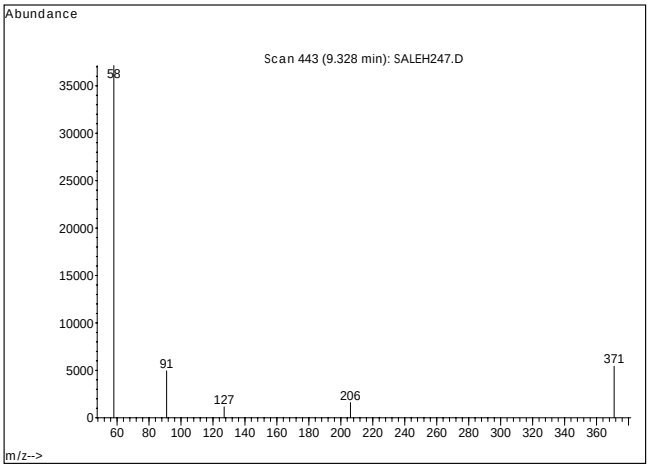

Fig. 11a) Mass spectrum of real sample after spiking by tamoxifen. conditions:microdrop volume:2.5µl, pH: 8, stirring rate :500rpm, extraction time:25 min, sample volume :6ml, temperature :50ºc, without salt addition.

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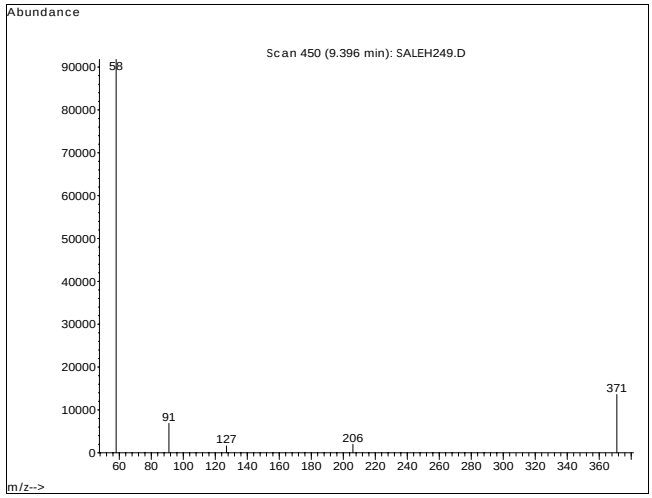

Fig 11 b) mass spectrum of bidistilled water after spiking by tamoxifen.conditions:microdrop volume:2.5µl, pH:8, stirring rate:500rpm,extraction time:25min, sample volume:6ml, temperature:50ºc, without salt addition.

The experimental results show that the relative recoveries are 50 %, table 3.

% Recovery	Relative peak area in bidistilled water	Relative peak area in real sample	Spiked with tamoxifen (µg/ml)
		0.001	0
43.3068	1.8362	0.7952	5
57.6918	2.4825	1.4322	10

Table 3 .results of recovery for calculations.

Conclusion

The method was based upon direct contact of the extracting microdrop with the sample solution . Influential parameters such as the type of solvent, extraction time, stirring rate, temperature, pH, microdrop volume, sample volume and ionic strength were optimized. The developed method is rather rapid, simple and reproducible. It is easy to use for the quantitative analysis of tamoxifen is easy and small volumes of sample and micro-scale size of organic extracting solvent are required.

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Protective effect of ketotifen on bleomycin-induced pulmonary fibrosis in rat

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ABSTRACT

Different ways for treatment of pulmonary fibrosis have been proposed. However; no definite treatment has been found yet. In the present Study, the effect of ketotifen has been investigated on bleomycin-induced pulmonary fibrosis in rats. Positive control group were given single intratracheal instillation of bleomycin. (7.5 IU/kg or 5 mg/kg). Control group received normal saline. Negative control group were given oral ketotifen (1mg/kg/day) for two weeks. Groups 4-6 received oral daily doses of ketotifen (0.05, 0.5 and 1mg/kg) 5 days before and 2 weeks after bleomycin (7.5 IU/kg) administration. Two weeks after such treatments, animals were killed and their lungs were removed, weighted and were prepared for histopathological studies and hydroxyproline determination. Lung weight in positive control group was significantly higher ($P< 0.01$) than control or ketotifen-treated groups. Histopathological examination of positive control group showed infiltration of the inflammatory cells into the alveolar space, deposition of collagen in interstitial associated with diffused pulmonary fibrosis. Ketotifen could reduce the inflammatory reactions and the fibrotic damage in lung tissue with a dose-dependent manner. Hydroxyproline and collagen value in positive control group was significantly higher ($P< 0.01$) than negative and saline control groups. In ketotifen-treated groups, such values were significantly less than positive control group. According to results of this study, we can suggest that ketotifen can protect the lung tissue against fibrogenic effect of bleomycin. It may exert its action by stabilization of mast cell membrane and prevention of the released of inflammatory factors during the process of lung fibrosis.

KEY WORDS: pulmonary fibrosis, bleomycin, ketotifen, rat

Introduction

A serious limitation in the use of the DNA-cleaving, antitumoral-antibiotic, bleomycin during chemotherapy is pulmonary toxicity. Lung injury induced by bleomycin is characterized by an increased deposition of interstitial extracellular matrix proteins in the alveolar wall that compromises respiratory function [1]. An increase in actin-containing non-muscle cells such as fibroblasts and myofibroblasts in fibrotic lung tissue have been described [1,2]. Such increase contribute to the excess production of collagen fibres in interstitum. Mechanism of bleomycin lung toxicity is not well understood, but many reports suggest that it can generate reactive oxygen species such as superoxide and hydroxyl radicals and certain cytokines e.g. TNFα [3]. Several drugs have been tested in animal models to prevent the pulmonary toxicity of bleomycin, but have not led to a useful clinical treatment because of their adverse effects on other tissues. Antioxidant and anti inflammatory agents have been tested to prevent the cell damages by free radicals [4,5].

Ketotifen is an antihistamine agent with prolonged preventive effect on the frequency and intensity of the asthma attacks. Its mechanism of action ultimately is associated with reduction of histamine, serotonin and other mast cell mediators release such as leukotrienes, prostaglandins and cytokines, and simultaneous selective blocking of the H1-receptors. As a result of the latter effects the level of the cell cAMP in enhanced and eosinophilic infiltration is inhibited [6]. With regard to the above effects, in this study the effect of ketotifen has been investigated on bleomycin-induced lung fibrosis in animal model.

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Materials and Methods

Male NMRI rats weighing 280-320g were used during the study. The animals were kept on standard food pellet and tap-water *ad libitum*. The rats were housed in polycarbonate cages (5 animals per cage) and kept in an air-conditioned animal room at a temperature of 23±3 °C, and a relative humidity of 50±5%. The animal room was on a 12h light/dark cycle.

Positive control group were given single intratracheal instillation of bleomycin. (7.5 IU/kg or 5 mg/kg in 0.2ml saline). Placebo group received the same volume of normal saline. Negative control group were given oral ketotifen (1 mg/kg) daily for two weeks. Tests groups received oral daily doses of ketotifen (0.05, 0.5 and 1mg/kg) 5 days before and 2 weeks after single intratracheal bleomycin (7.5 IU/kg) administration. Two weeks after such treatments, animals were killed and their lungs were removed, weighted and were sampled for histopathological studies and hydroxyproline determination.

Left lobe of lung was fixed in 10% formalin. Lung tissue was processed and cut into 5micron sections then stained with H&E. Histopathological changes due to bleomycin and the effect of ketotifen was investigated by light microscopy. Right lobe of lung was used for hydroxyproline determination. Tissue samples were homogenised and processed according to the earlier discussed method. Hydroxyproline content of lung tissue was determined by colorimetric method as described by Edwards and O'Brien [7]. Hydroxyproline was extracted from collagen and oxidised to pyrrole by chloramine-T, then it can produce colour with *para*-dimethylbenzaldehyde. The absorbance was measured at 500 nm to determine hydroxyproline content (mg/g tissue).

Statistical analysIs were performed using one way ANOVA followed by multiple comparison with Dunnet's test. The differences were considered significant when $p<0.05$.

Results

Histological examination of bleomycin-treated lung sections showed marked infiltaration of infalmmatory cells into the alveolar spaces and interstitum, associated with septal thickening and large amount of collagen in interstitial area indicating pulmonary fibrosis. The lesions were diffuse in nature involving many areas of the lung. Control lungs had normal parenchyma with alveolar septum (figs 1&2). Administration of ketotifen could significantly reduce the fibrotic lesions, due to bleomycin in a dose-dependent manner (figs 3&4). Lung weights of bleomycin treated was significantly higher than control groups (fig 5). Bleomycin significantly elevated hydroxyproline and collagen content of lung tissue compared to normal lungs. Hydroxyproline and collagen were significantly reduced after ketotifen treatment to a value similar to control groups. The effect of ketotifen was dose dependent (figs 4-7).

Discussion

In the present study we evaluated the effect of ketotifen on bleomycin-induced pulmonary fibrosis. Both biochemical and histological results of this work confirmed that ketotifen is able to decrease the fibrotic effect of bleomycin. Similar results have not been reported before but beneficial effect of ketotifen on the development of fibrosis in alkali burns of the esophagus has been observed [8]. We have stdied the effect of sodium cromolyn on paraquat-induced lung fibrosis which had a promising result [9]. Mast cell stabilizing effect of ketotifen and its inhibitory action on eicosanids and immune system may be responsible for ketotifen effects on bleomycin-induced pulmonary fibrosis [10,11]. With the advantages of low adverse effects of ketotifen we may suggest the trail of ketotifen in patients exposed to fibrogenic agents to reduce the chance of pulmonary fibrosis development.

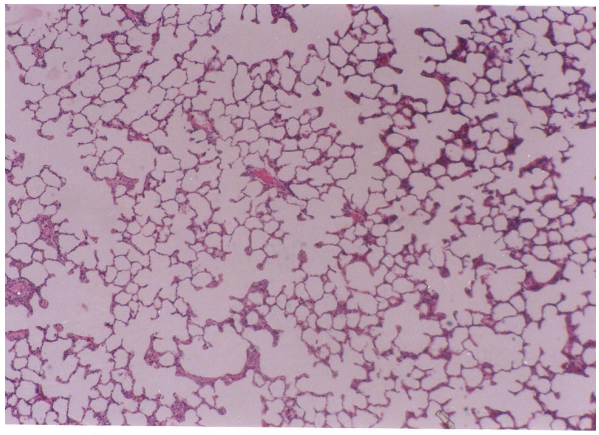

Fig. 1: Photomicrograph of normal lung section. No septal thickening or inflammatory cells in alveolar spaces are seen (H&E ×50).

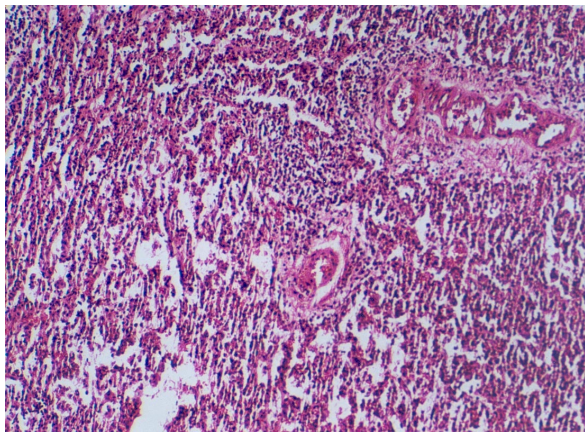

Fig. 2: Photomicrograph of bleomycin-treated rat lung section (H&E X40).

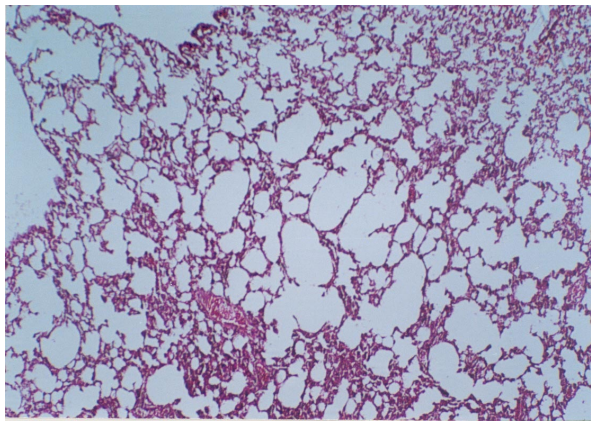

Fig 3: Photomicrograph of rat lung section which received bleomycin and treated with 0.5mg/kg ketotifen (H&E X40).

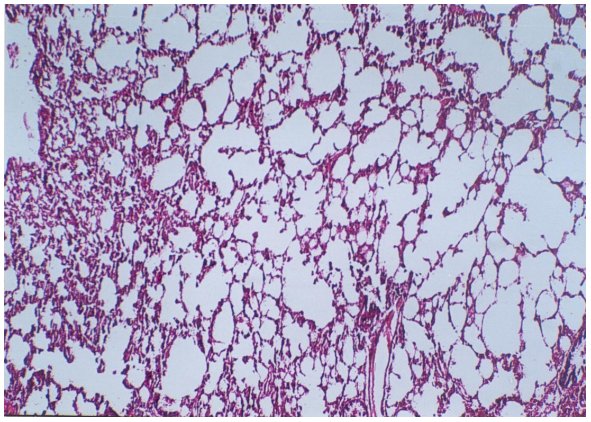

Fig 4: Photomicrograph of rat lung section which received bleomycin and treated with 1mg/kg ketotifen (H&E X40).

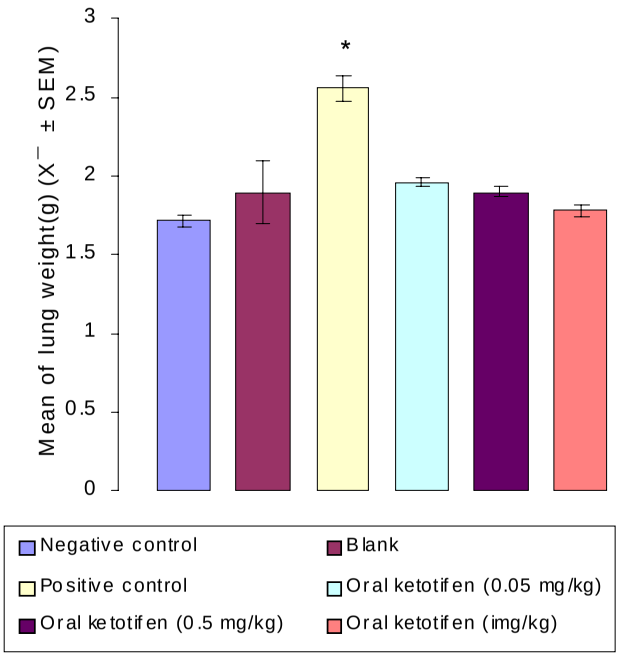

Fig 5: Comparison of lung weights in different groups of rats. Values significantly different from other groups are indicated * (p<0.001).

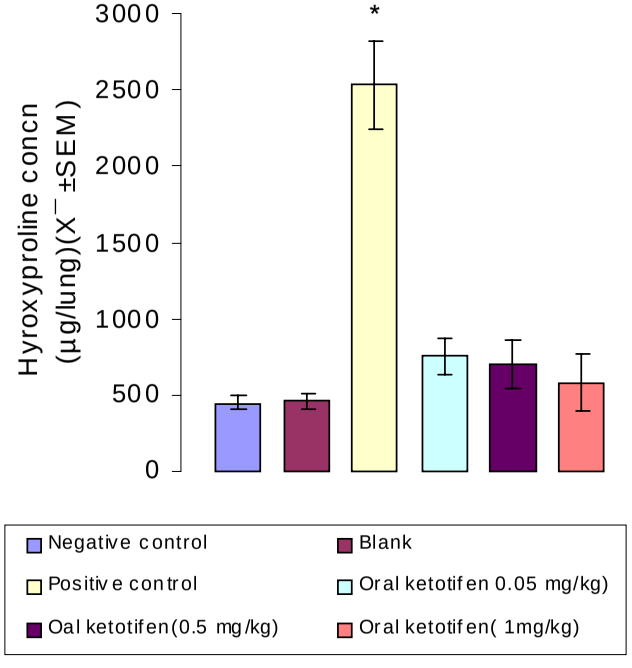

Fig. 6: Comparison of lung hydroxyproline in different groups of rats. Values significantly different from other groups are indicated * (p<0.001)

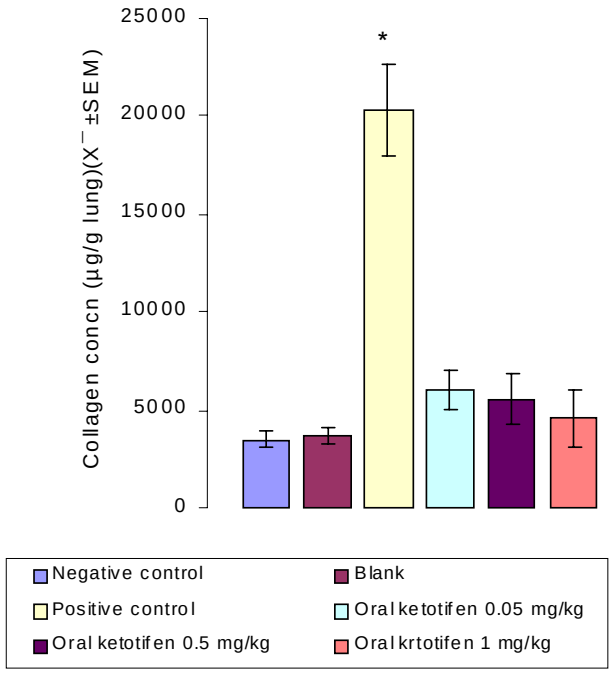

Fig 7: Comparison of lung collagen in different groups of rats. Values significantly different from other groups are indicated * (p<0.001)

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Experimental Design for the Calibration, Validation and Determination of Paracetamol and Caffeine by Direct and Derivative Uv-visible Spectrophotometry

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ABSTRACT

Paracetamol is an acylated aromatic amide, which was firstly introduced into medicine as an antipyretic/analgesic by Von Mering in 1893 and has been in use as an analgesic for home medication and caffeine is a xanthine alkaloid compound that acts as a stimulant in humans. Paracetamol and caffeine are frequently associated in many commercial formulations because caffeine increases the analgesic character of paracetamol.

The purpose of this study was to compare two techniques of data analysis: Analysis of variance and linear multiple regression, as used to evaluate an experimental design for matrix calibration to determine paracetamol and caffeine in mixtures. Paracetamol and caffeine are simultaneously determined using mathematical equations.

KEYWORDS: Caffeine; Paracetamol; Experimental design; ANOVA, Multiple linear regression

Introduction

Paracetamol (PCT) has been in use as an analgesic for home medication for over 30 years and is accepted as a very effective treatment for the relief of pain and fever in adults and children. PCT is now the most widely accepted antipyretic and analgesic in the world.

PCT, also known as acetaminophen (*N*-acetyl-*p*-aminophenol, 4-acetamidophenol), is an acylated aromatic amide (Figure 1); it is a major ingredient in numerous cold and flu medications and many prescription analgesics. It is remarkably safe in standard doses, but, because of its wide availability, deliberate or accidental overdoses are not uncommon. PCT, unlike other common analgesics such as aspirin and ibuprofen, has no anti-inflammatory properties, and so it is not a member of the class of drugs known as *non-steroidal anti-inflammatory drugs* or NSAIDs. In normal doses, PCT does not irritate the lining of the stomach or affect blood coagulation, the kidneys, or the fetal ductus arteriosus. Like NSAIDs and unlike opioid analgesics, PCT has not been found to cause euphoria or alter mood in any way. PCT and NSAIDs have the benefit of bearing no risk of addiction, dependence, tolerance and withdrawal. Data about chemical, physical and biopharmaceutical properties are easily available.

At normal therapeutic doses, PCT is metabolised very fast and completely by undergoing glucuronidation and sulphation to inactive metabolites that are eliminated in the urine. However, PCT higher doses produce toxic metabolite accumulation that causes hepatocyte death. Acetaminophen overdose is a frequent cause of fulminating hepatic failure in Europe and US. Based on the aforementioned observations the main objective was to carry out the development of more efficient analytical techniques, destined to quality control of one of the medicaments more widely used. Several methods have been utilised for its determination in pure form, formulation and combination with other substances; mainly volumetric, polarographic, UV–vis spectrophotometric, fluorimetric, chromatographic and many others [1].

Caffeine (CAF) is a xanthine alkaloid compound (Figure 2) that acts as a stimulant in humans. The word comes from the Italian term for coffee, caffè. CAF is also called guaranine when found in guarana, mateine when found in mate, and theine when found in tea, although these terms are not considered valid chemical names. It is found in the leaves and beans of the coffee plant, in tea, yerba mate, and

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guarana berries, the , the Yaupon Holly, and in small quantities in cocoa. Overall, CAF is found in the beans, leaves, and fruit of over 60 plants, where it acts as a natural pesticide that paralyzes and kills certain insects feeding on the plants.

In humans, CAF is a central nervous system stimulant, having the effect of temporarily warding off drowsiness and restoring alertness. Beverages containing CAF, such as coffee, tea, soft drinks and energy drinks enjoy great popularity; CAF is the world's most widely consumed psychoactive substance, but unlike most other psychoactive substances, it is legal and unregulated in nearly all jurisdictions. CAF is used both recreationally and medically to reduce physical fatigue and restore mental alertness when unusual weakness or drowsiness occurs. CAF stimulates the central nervous system first at the higher levels, resulting in increased alertness and wakefulness, faster and clearer flow of thought, increased focus, and better general body coordination, and later at the spinal cord level at higher doses. There are many methods for the simultaneous determination of PCT and CAF including spectrophotometric [2-11], spectrofluorimetric [12], electroanalytical [13-14], high-performance liquid chromatography [15-16], capillary electro-chromatography [17-18] and gas chromatography techniques [19]. Chromatographic methods require expensive instrumentation and are relatively highly time-consuming. Although, spectrophotometric methods are simpler and faster, the simultaneous determination of both analytes is not possible by conventional direct UV absorption measurements, because of the spectral overlap.

The main problem of spectrophotometric multicomponent analysis is the simultaneous determination of two or more compounds in the same mixtures without preliminary separation. Several spectrophotometric determination methods have been used for resolving mixtures of compounds with overlapping spectra. When these methods are compared with each other, the range of application of derivative spectrophotometry is more reliable with respect to utility and sensitivity than normal spectrophotometry [20].

Computer-controlled instrumentation and multivariate calibration methods are playing a very important role in the multi-component analysis of mixtures by UV/VIS spectrophotometry. Both approaches are useful for the resolution of band overlapping in the quantitative analysis. In general, a multivariate calibration model is constructed from instrumental response data collected for a set of multi-component samples of known concentrations with respect to the analytes of interest. Each method needs this calibration step, followed by a prediction step in which the results of the calibration are used to determine the component concentrations from the sample spectrum.

The aim objective of this work is the establishment of a calibration matrix and its validation to determine paracetamol and caffeine mixtures by direct and derivative UV-visible spectroscopic methods making use of the experimental design. Statgraphics plus 5.1 programs has been used for this purpose.

Experimental

Reagents

All solutions were prepared from analytical reagent-grade chemicals using deionised water.

Paracetamol (Fluka) and caffeine (Merck) stock solutions (1000 mg L^{-1}) were prepared by directly dissolving the drug in deionised water. Only freshly prepared solutions of paracetamol were used due to the low stability. The caffeine solution was stable for at least four weeks at 4 – 5ºC. Work solutions were prepared fresh daily by appropriate dilution with deionised water.

Instrumentation and Sofware

A Varian Cary 50 Spectrophotometer was used for absorbance measurements. The spectra were obtained using a cell of 1 cm optical path length.

Statgraphics plus 5.1 programs has been used for experimental design and statistical studies.

Preliminary study

The spectral features of both analytes in homogeneous solutions of PCT and CAF were previously established. Because the scans of the analytes overlapped, it was impossible to conduct a simultaneous determination by conventional spectrophotometric measurements without significant errors.

Calibration matrix and data analysis

The calibration matrix was statistically designed over the concentrations ranges of 0.5-6 μg mL^{-1} of each analyte. A multi-level factorial design (2^4) has been used for the calibration matrix design. The design has been carry out in two block and the order of experiments have been randomize for protect they from occult variables. 32 synthetic mixtures of CAF and PCT were employed (according to Table 1). The concentration ranges were selected within the previously established linear calibration range for each compound.

Analysis of variance and multiple linear regression techniques has been applied to the data analysis obtained experimentally from absorption spectra, first, second, third and fourth derivative spectra.

Results and Discussion

Calibration graphs

Figures 1-5 show the absorption spectra and the corresponding first, second, third and four derivative spectra of solutions of PCT, CAF and a mixture of both analytes, respectively. As can be seen, figure 1 shows that the absorption spectra of PCT and CAF in binary mixture are strongly overlapping in the wavelength range of 190-300 nm. The quantitative analysis of the content of binary mixtures consisting of the above active compounds with overlapping spectra is not always possible by using conventional spectrophotometric methods.

Table 2 contains the figures of merit of the calibration graphs at the wavelength selected for PCT and CAF. A statistical analysis of the experimental data by fitting the overall least-squares line according to $y = a + bx$ [21] was performed. Good linearity was found in the concentration range 0.5-6 μg mL^{-1} for PCT and CAF in all cases.

Factorial design

Two level factorial designs have many advantages in analytical procedures. In this method, there are comparisons available for each main effect on the experimental results. The total number of runs in factorial design is much less as compared to the "one variable at a time" approach. Estimation of the interaction of effects is an additional advantage over the "one variable at a time" approach.

In this work, a multi-level factorial design [22], 2^4, involving 32 runs were used to calibration. The factors and their levels are shown in Table 1.

The significance of the effects was checked by analysis of the variance (ANOVA) and using *P*-value significance levels. This value represents the probability of the effect of a factor being due solely to random error. Thus, if the *P*-value is less than 5%, the effect of the corresponding factor is significant. Table 3 shows the results obtained of these studies for the all selected wavelengths. Also, the ANOVA results for lead produced the graphs showing the influence of main effects represented in figure 6, interaction plot in figure 7 and standardized Pareto chart in figure 8.

On the other hand, the results obtained fit the equations display in Table 4 for each selected signal.

With respect to the linear regression model, the results obtained were similar to the ANOVA after to eliminate the no significant effects. In table 5 are showed the linear equations for this study.

Validation of procedure

In the prediction step, the constructed calibration models were applied to the fifteen mixtures of analytes in the working concentration range and their results were given in Tables 6-8. These values indicate good predictive ability of the established models in all cases.

Determination of CAF and PCT in synthetic mixtures

In order to check for potential synergistic effects of the mixtures of two compounds, various synthetic mixtures were readily resolved by using pertinent regression equations for different adjusted models. The analytical results are listed in Table 9. As can be seen, mixtures of the two analytes can be resolved with satisfactory results.

Conclusions

Caffeine and paracetamol are very interesting compounds from the point of view of their applications.

Table 1. Calibration Matrix

Row	Block	Caffeine (ppm)	Paracetamol (ppm)
1	1	1	2,7
2	1	6	1
3	1	2,7	6
4	1	2,7	4,3
5	1	4,3	6
6	1	4,3	2,7
7	1	6	6
8	1	6	2,7
9	1	1	4,3
10	1	2,7	2,7
11	1	4,3	4,3
12	1	6	4,3
13	1	4,3	1
14	1	1	1
15	1	1	6
16	1	2,7	1
17	2	1	2,7
18	2	1	4,3
19	2	6	2,7
20	2	4,3	2,7
21	2	6	6
22	2	4,3	4,3
23	2	6	4,3
24	2	1	6
25	2	4,3	1
26	2	1	1
27	2	6	1
28	2	2,7	4,3
29	2	2,7	6
30	2	2,7	1
31	2	4,3	6
32	2	2,7	2,7

Table 2. Figures of merit

Selected wavelength	Parameter							
	Calibration graph							
	Linear dynamic range (µg mL^{-1})		Intercept		Slope coefficient		Correlation	
	PCT	CAF	PCT	CAF	PCT	CAF	PCT	CAF
Absorbance at 205 nm	0.5 -6	0.5 - 6	0.267	0.249	0.123	0.153	0.999	0.995
Absorbance at 210 nm	0.5 -6	0.5 -6	0.209	0.188	0.061	0.138	0.996	0.995
Absorbance at 240 nm	0.5 -6	0.5 -6	0.049	0.044	0.076	0.016	0.999	0.925
Absorbance at 275 nm	0.5 -6	0.5 -6	0.014	0.004	0.015	0.054	0.976	0.995
1st derivative at 204-206 nm	0.5 -6	0.5 -6	0.004	0	0.003	0.010	0.989	0.999
2nd derivative at 205 nm	0.5 -6	0.5 -6	0.001	0	0.001	0.004	0.989	0.999
2nd derivative at 220 nm	0.5 -6	0.5 -6	0	0	0.001	0.002	0.997	0.996
3th derivative at 204-206 nm	0.5 -6	0.5 -6	0.003	0	0.003	0.009	0.989	0.999
3th derivative at 219-221 nm	0.5 -6	0.5 -6	0	0	0.002	0.005	0.997	0.996
4th derivative 205-207 nm	0.5 -6	0.5 -6	0.003	0	0.002	0.009	0.989	0.999
4th derivative at 220-222 nm	0.5 -6	0.5 -6	0	0	0.002	0.005	0.997	0.996

Table 3. Analysis of variance

Selected wavelength	P-value					R-square (%)
	Source					
	A: CAF	B: PCT	AA	AB	BB	
Absorbance at 205 nm	0,0000	0,0000	0,0168	0,0813	0,3354	99.7696
Absorbance at 210 nm	0,0000	0,0000	0,0552	0,6713	0,3743	99.9873
Absorbance at 240 nm	0,0000	0,0000	0,5974	0,6626	0,2148	98.5622
Absorbance at 275 nm	0,0000	0,0000	0,1003	0,5921	0,5979	99.5012
1st derivative at 204-206 nm	0,0000	0,0760	0,8565	0,8748	0,1822	96.4653
2nd derivative at 205 nm	0,0000	0,0783	0,9086	0,8864	0,1942	96.4459
2nd derivative at 220 nm	0,0000	0,0000	0,1884	0,7556	0,1359	97.2241
3th derivative at 204-206 nm	0,0000	0,0785	0,7734	0,8664	0,1635	96.4593
3th derivative at 219-221 nm	0,0000	0,0000	0,1658	0,2284	0,3196	99.3537
4th derivative 205-207 nm	0,0000	0,1338	0,7418	0,9183	0,1512	96.3019
4th derivative at 220-222 nm	0,0000	0,0000	0,1624	0,2385	0,3393	99.312

Table 4. Regression equations for different adjusted models

Selected wavelength	Equations for dependent variable (Y)
Absorbance at 205 nm	$Y = 0,177852 + 0,277608 \cdot [caffeine] + 0,132392 \cdot [paracetamol] - 0,00430036 \cdot [caffeine]^2 - 0,00248285 \cdot [caffeine] \cdot [paracetamol] - 0,00164884 \cdot [paracetamol]^2$
Absorbance at 210 nm	$Y = 0,138445 + 0,24026 \cdot [caffeine] + 0,0701429 \cdot [paracetamol] - 0,00331996 \cdot [caffeine]^2 - 0,000577023 \cdot [caffeine] \cdot [paracetamol] - 0,00149287 \cdot [paracetamol]^2$
Absorbance at 240 nm	$Y = 0,0134465 + 0,0376753 \cdot [caffeine] + 0,0807593 \cdot [paracetamol] - 0,000646168 \cdot [caffeine]^2 - 0,00043436 \cdot [caffeine] \cdot [paracetamol] - 0,00153743 \cdot [paracetamol]^2$
Absorbance at 275 nm	$Y = -0,0203716 + 0,101123 \cdot [caffeine] + 0,0214966 \cdot [paracetamol] - 0,00145945 \cdot [caffeine]^2 - 0,00037788 \cdot [caffeine] \cdot [paracetamol] - 0,000456774 \cdot [paracetamol]^2$
1st derivative at 204-206 nm	$Y = -0,000319692 + 0,0163915 \cdot [caffeine] - 0,00525021 \cdot [paracetamol] - 0,0000746435 \cdot [caffeine]^2 + 0,0000529153 \cdot [caffeine] \cdot [paracetamol] + 0,000560383 \cdot [paracetamol]^2$
2nd derivative at 205 nm	$Y = -0,000120038 + 0,00608654 \cdot [caffeine] - 0,00192437 \cdot [paracetamol] - 0,0000178253 \cdot [caffeine]^2 + 0,0000180527 \cdot [caffeine] \cdot [paracetamol] + 0,000204991 \cdot [paracetamol]^2$
2nd derivative at 220 nm	$Y = 0,00153008 + 0,00250852 \cdot [caffeine] - 0,00016924 \cdot [paracetamol] + 0,0000958111 \cdot [caffeine]^2 - 0,0000181554 \cdot [caffeine] \cdot [paracetamol] + 0,00010918 \cdot [paracetamol]^2$
3th derivative at 204-206 nm	$Y = -0,000288684 + 0,001374 \cdot [caffeine] - 0,00448149 \cdot [paracetamol] - 0,0000980392 \cdot [caffeine]^2 + 0,0000466327 \cdot [caffeine] \cdot [paracetamol] + 0,000483512 \cdot [paracetamol]^2$
3th derivative at 219-221 nm	$Y = 0,00104157 + 0,00650389 \cdot [caffeine] + 0,000795365 \cdot [paracetamol] + 0,000108066 \cdot [caffeine]^2 - 0,0000760973 \cdot [caffeine] \cdot [paracetamol] + 0,0000768717 \cdot [paracetamol]^2$
4th derivative 205-207 nm	$Y = -0,000430223 + 0,0136512 \cdot [caffeine] - 0,00439245 \cdot [paracetamol] - 0,000112522 \cdot [caffeine]^2 + 0,0000284878 \cdot [caffeine] \cdot [paracetamol] + 0,000500223 \cdot [paracetamol]^2$
4th derivative at 220-222 nm	$Y = 0,000983676 + 0,00634857 \cdot [caffeine] + 0,000803854 \cdot [paracetamol] + 0,000110294 \cdot [caffeine]^2 - 0,0000753364 \cdot [caffeine] \cdot [paracetamol] + 0,0000746435 \cdot [paracetamol]^2$

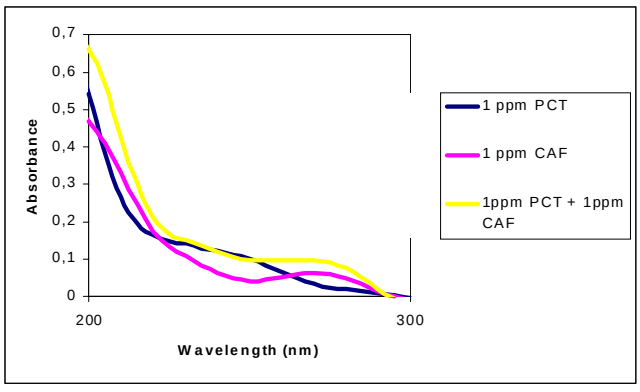

Fig.1: Absorption spectra of PCT, CAF and mixtures

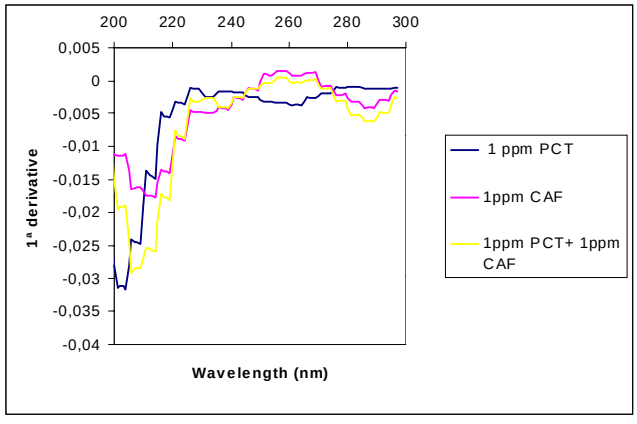

Fig.2: First derivative spectra of PCT, CAF and mixtures

Table 5. Linear regression models

Selected wavelength	Equations for dependent variable (Y)	Correlation coefficient (%)
Absorbance at 205 nm	*Y = 0,260649 + 0,238815 · [caffeine] + 0,11216· [paracetamol]*	99.6648
Absorbance at 210 nm	*Y = 0,18789 + 0,215001 · [caffeine] + 0,0576733· [paracetamol]*	99.6242
Absorbance at 240 nm	*Y = 0,037994 + 0,0316319 · [caffeine] + 0,068477· [paracetamol]*	98.1826
Absorbance at 275 nm	*Y = 0,00112976 + 0,0895845 ·[caffeine] + 0,0169766 · [paracetamol]*	99.3347
1st derivative at 204-206 nm	*Y = -0,00524484 + 0,0160542 ·[caffeine] - 0,00114233 · [paracetamol]*	96.1457
2nd derivative at 205 nm	*Y = -0,00198918 + 0,00602495 ·[caffeine] - 0,000426252 · [paracetamol]*	96.1793
2nd derivative at 220 nm	*Y = -0,0000524673 + 0,00311566 ·[caffeine] + 0,000531477· [paracetamol]*	96.7307
3rd derivative at 204-206 nm	*Y = -0,00425402 + 0,013217 ·[caffeine] - 0,00093369· [paracetamol]*	95.9594
3rd derivative at 219-221 nm	*Y = 0,000345387 + 0,00699401 ·[caffeine] + 0,00106713 · [paracetamol]*	98.8766
4th derivative 205-207 nm	*Y = -0,0041929 + 0,0129633 ·[caffeine] - 0,000791183· [paracetamol]*	95.7309
4th derivative at 220-222 nm	*Y = 0,00027817 + 0,00685695 ·[caffeine] + 0,00106268· [paracetamol]*	98.8222

Table 9. Resolution of synthetic PCT-CAF mixtures

Concentration added (µg mL^{-1})		Concentration found (µg mL^{-1})	
Caffeine	**Paracetamol**	**Caffeine**	**Paracetamol**
1	1	0,9 ± 0,1	1,1 ± 0,1
1	2	1,0 ± 0,1	1,8 ± 0,1
1	3	1,0 ± 0,1	2,9 ± 0,2
2	2	1,8 ± 0,3	2,5 ± 0,3
3	3	2,9 ± 0,5	3,1 ± 0,1
2	4	1,5 ± 0,2	4,2 ± 0,3
3	6	3,0 ± 0,3	6,0 ± 0,3
2	1	1,9 ± 0,2	1,2 ± 0,1
3	1	3,0 ± 0,1	0,9 ± 0,1
1	4	0,95 ± 0,1	4,0 ± 0,1
1	5	0,9 ± 0,1	5,1 ± 0,2
1	6	0,9 ± 0,2	6,2 ± 0,1
4	1	3,7 ± 0,5	1,0 ± 0,2
5	1	4,5 ± 0,2	1,1 ± 0,1
6	1	5,0 ± 0,5	1,1 ± 0,1

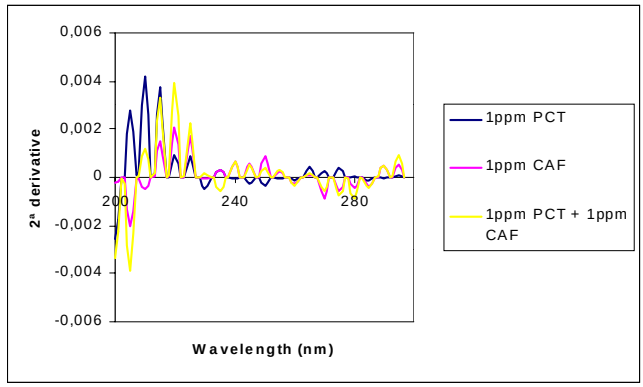

Fig.3: Second derivative spectra of PCT, CAF and mixtures

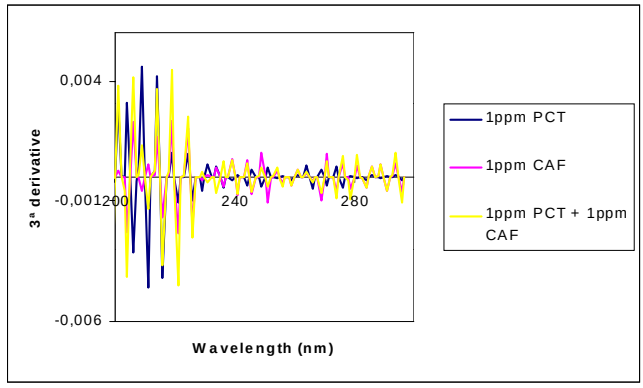

Fig.4: Thirst derivative spectra of PCT, CAF and mixtures

Table 6. Results for validation of procedure: Absorbance measurements

CAF*	PCT*	Selected wavelength (nm)										
		205			210			240			275	
		Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA
1	1	0,571	0,579419	0,611625	0,417	0,443458	0,460565	0,160	0,129263	0,138103	0,093	0,0999542
1	2	0,704	0,704382	0,723784	0,481	0,508545	0,518238	0,248	0,204976	0,20658	0,109	0,119703
1	3	0,864	0,826046	0,835944	0,566	0,570647	0,575911	0,337	0,277617	0,275057	0,133	0,138537
2	2	0,873	0,964123	0,9626	0,628	0,737692	0,733239	0,264	0,239844	0,238212	0,168	0,215692
3	3	1,169	1,33196	1,31357	0,836	1,02115	1,00591	0,371	0,345189	0,338321	0,248	0,326841
2	4	1,152	1,19919	1,18692	0,776	0,857755	0,848585	0,440	0,381176	0,375166	0,209	0,251692
3	6	1,575	1,66227	1,65005	1,044	1,18607	1,17893	0,617	0,542046	0,243751	0,299	0,375597
2	1	0,736	0,841643	0,85044	0,560	0,673182	0,675565	0,178	0,164566	0,169735	0,152	0,196321
3	1	0,898	1,09527	1,08926	0,705	0,896265	0,890566	0,199	0,198576	0,201367	0,211	0,289769
1	4	0,990	0,944413	0,948104	0,621	0,629763	0,633584	0,421	0,347176	0,343534	0,147	0,156459
1	5	1,138	1,05948	1,06026	0,699	0,685893	0,691258	0,507	0,413664	0,412011	0,167	0,173466
1	6	1,276	1,17125	1,17242	0,770	0,739037	0,748931	0,586	0,477077	0,480488	0,179	0,189561
4	1	1,070	1,34029	1,32807	0,860	1,11271	1,10557	0,215	0,231294	0,232999	0,269	0,380299
5	1	1,222	1,57671	1,56689	0,992	1,32251	1,32057	0,229	0,262719	0,26463	0,323	0,467909
6	1	1,381	1,80453	1,8057	1,127	1,52568	1,53557	0,249	0,292852	0,296262	0,380	0,5526

* Concentrations in $\mu g\ mL^{-1}$

Table 7. Results for validation of procedure: First and second derivative measurements

CAF *	PCT *	Selected wavelength (nm)								
		First derivative			Second derivative					
		204-206			205			220		
		Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	A v R
1	1	0,0029	0,0113603	0,00966708	0,0011	0,00424735	0,00360952	0,0030	0,00405619	
1	2	0,0001	0,00784415	0,00852475	0,0001	0,002956	0,00318326	0,0068	0,00419634	
1	3	0,0034	0,00544877	0,00738242	0,0013	0,00207463	0,00275701	0,0046	0,00455485	
2	2	0,0112	0,0241176	0,024579	0,0042	0,00902516	0,00920821	0,0127	0,00695599	
3	3	0,0197	0,0379522	0,0394909	0,0073	0,0142134	0,0148069	0,0085	0,0102294	
2	4	0,0022	0,0205534	0,0222943	0,0008	0,00770852	0,00835571	0,0075	0,00785505	
3	6	0,0158	0,0378082	0,0360639	0,0059	0,0141375	0,0135281	0,0104	0,0125062	
2	1	0,0147	0,0275808	0,0257213	0,0055	0,0102985	0,00963446	0,0053	0,006834	
3	1	0,0259	0,0436521	0,0417756	0,0096	0,0163139	0,0156594	0,0074	0,00380342	
1	4	0,0069	0,00417416	0,0062401	0,0026	0,00160325	0,00233076	0,0043	0,00513171	
1	5	0,0120	0,00402031	0,00509777	0,0045	0,00154185	0,00190451	0,0062	0,00592694	
1	6	0,0102	0,00498723	0,00395545	0,0038	0,00189043	0,00147826	0,0064	0,00694052	
4	1	0,0373	0,059574	0,0578298	0,0139	0,0222937	0,0216844	0,0096	0,0129645	
5	1	0,0488	0,0753467	0,0738841	0,0181	0,0282379	0,0277093	0,0114	0,0163171	
6	1	0,0632	0,0909701	0,0899383	0,0235	0,0341464	0,0337342	0,0142	0,0198614	

* Concentrations in μg mL^{-1}

Table 8. Results for validation of procedure: Third and fourth derivative measurements

CAF*	PCT*	Selected wavelength (nm)										
		Third derivative						Fourth derivative				
		204-206			219-221			205-207			220-22	
		Exp. value	Adjusted value ANOVA	Adj. value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA	Adjusted value Regression	Exp. value	Adjusted value ANOVA
1	1	0,0025	0,00940198	0,00802927	0,0037	0,00844967	0,00840653	0,0027	0,00924473	0,00797918	0,0064	0,0082457
1	2	0,0001	0,00641766	0,00709558	0,0147	0,00939955	0,00947365	0,0005	0,00638143	0,00718799	0,0153	0,00919815
1	3	0,0028	0,00440036	0,00616189	0,0099	0,0105032	0,0105408	0,0019	0,00451858	0,00639681	0,0098	0,0102999
2	2	0,0094	0,0199568	0,0203126	0,0272	0,0160754	0,0164677	0,0098	0,0197521	0,0201513	0,0283	0,0157269
3	3	0,0165	0,0313759	0,0325959	0,0182	0,0239189	0,0245288	0,0170	0,0310918	0,0323233	0,0179	0,0234274
2	4	0,0018	0,0169825	0,0184452	0,0160	0,0182842	0,0186019	0,0027	0,0170838	0,0185689	0,0158	0,017929
3	6	0,0132	0,031406	0,0297948	0,0224	0,0276957	0,0277302	0,0145	0,0316768	0,0299498	0,0221	0,0271763
2	1	0,0123	0,0228945	0,0212463	0,0114	0,0152017	0,0154005	0,0124	0,0225869	0,0209424	0,0112	0,0148498
3	1	0,0217	0,036191	0,0344632	0,0159	0,0221698	0,0223946	0,0215	0,0357039	0,0339057	0,0156	0,0216745
1	4	0,0058	0,00335009	0,0052282	0,0092	0,0117605	0,0116079	0,0045	0,00365618	0,00560563	0,0098	0,0115509
1	5	0,0101	0,00326684	0,00429451	0,0134	0,0131717	0,012675	0,0085	0,00379423	0,00481445	0,0133	0,0129512
1	6	0,0085	0,00415062	0,00336082	0,0138	0,0147365	0,0137422	0,0067	0,00493271	0,00402326	0,0137	0,0145008
4	1	0,0313	0,0492914	0,0476802	0,0205	0,029354	0,0293886	0,0308	0,048596	0,046869	0,0201	0,0287198
5	1	0,0409	0,0621957	0,0608972	0,0245	0,0367544	0,0363826	0,0402	0,061263	0,0598322	0,0240	0,0359857
6	1	0,0530	0,074904	0,0741142	0,0304	0,044371	0,0433766	0,0523	0,0737049	0,0727955	0,0298	0,0434721

* Concentrations in μg mL^{-1}

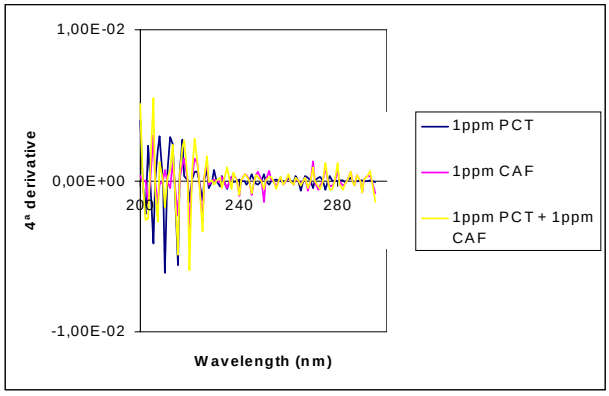

Fig.5: Fourth derivative spectra of PCT, CAF and mixtures

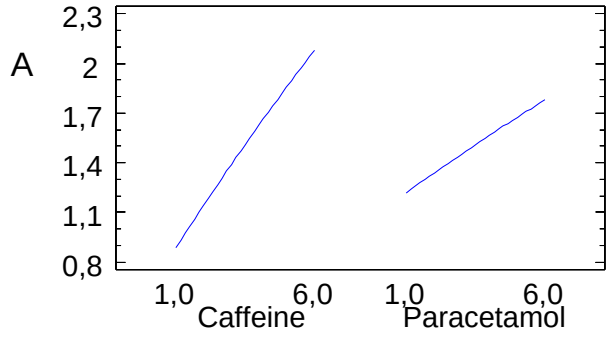

Fig.6: Graphic of main effects for absorbance measurements (l= 205 nm)

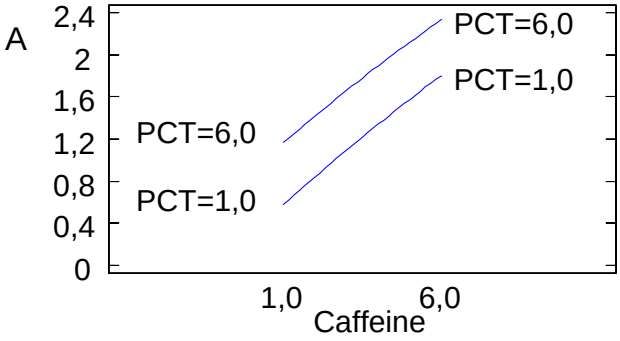

Fig.7: Interaction graphic for absorbance measurements (l=205 nm)

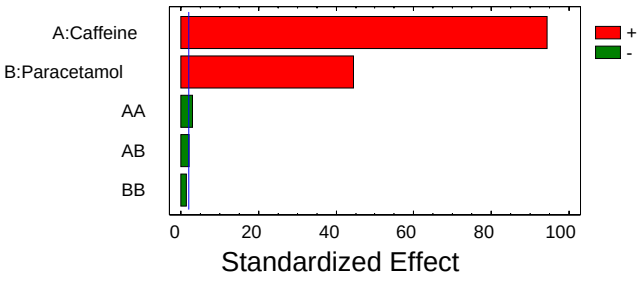

Fig.8: Pareto diagram for absorbance measurements (l= 205 nm)

The individual caffeine and paracetamol calibrations have been obtained, using signals in absorbance, and first, second, third a fourth derivatives.

Statgraphics plus 5.1 program has been used for the creation of the calibration matrix, using a factorial design multilevel (2^4) that consists of 32 executions. The data collected have been analyzed by means of ANOVA and multiple regression analysis.

The validation of the procedure has been carried out by means of analysis ANOVA and of multiple regression for each one of the collected data, this it is absorbance, first, second, third and fourth derivatives.

Finally, the analysis of synthetic samples shows that obtain good percentage of recovery in all the studied cases.

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Studies on Diclofenac Sodium Loaded Ethyl Cellulose Microcapsules

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ABSTRACT

The objective of the present study is to analyze the kinetics of in vitro release of Diclofenac Sodium (DFS) from Ethyl Cellulose (EC) microcapsules. DFS, a phenyl acetate non steroidal anti-inflammatory agent was microencapsulated by coacervation-phase separation technique employing ethyl cellulose - toluene - petroleum ether system. Microcapsules using five ratios of EC and DFS: 1:9, 1:4, 2:3, 3:2, and 3:1 were prepared and studied. The microcapsules obtained were free flowing. Slow release of drug from microcapsules was observed when compared to unencapsulated drug. The drug release process from microcapsules followed first order kinetics was diffusion controlled, and decreased as the coat (EC) proportion increased. Linear relationship was observed between percent coat material and T_{50} values. These results indicated that EC can be used as rate controlling membrane and the non aqueous coacervation-phase separation technique employed is suitable for achieving Sustained Release.

KEY WORDS: Diclofenac Sodium, Ethyl cellulose, Coacervation, Phase separation, Microencapsulation

Introduction

Microencapsulation provides a simple and cost-effective way to enclose bioactive materials, such as drugs and cells, within a semi-permeable polymeric membrane for the purpose of protecting the bioactive materials and releasing the enclosed substances or their products in a controlled fashion [1]. The microcapsule coating can be chosen from a wide variety of natural and synthetic polymers [2, 3]. Microencapsulation by coacervation has wide commercial applications, including manufacture of sustained release dosage forms [4, 5]. Diclofenac sodium (DFS) is a widely used phenyl acetate non-steroidal anti-inflammatory agent in the treatment of rheumatic disorders. Because of its relative short biological half-life, the hazards of adverse gastro-intestinal reactions and chronic nature of treatment, and to improve patient compliance, sustained release dosage forms of DFS are needed [6, 7]. Ethyl cellulose (EC) is an inert, hydrophobic polymer and is essentially tasteless, odorless, colorless, non-caloric, and physiologically inert. It has been widely used in Microencapsulation [8]. In the present work, DFS was microencapsulated with EC by non aqueous phase separation induced by non solvent addition. Different EC: DFS ratios were tried and the resulting microcapsules were studied for drug content, microencapsulation efficiency, yield percentage, and *in-vitro* release characteristics.

Materials and Methods

Materials

Diclofenac Sodium (gift sample from Amoli Organics, Ahmedabad), Ethyl Cellulose (BDH) (having an ethoxyl content of 47.5% weight and a viscosity of 22 cp in a 5 % concentration, by weight, in a 80: 20 toluene-ethanol solution at 25^0 C), Toluene (BDH) (109^0-112^0 C), Petroleum ether (Glaxo) (60^0-80^0 C).

Methods

Preparation of Microcapsules

DFS was microencapsulated with EC by a reported method [9]. Briefly, EC was dissolved in 50 ml of warm toluene to form homogeneous polymer solution. Core material, DFS was then added to polymer solution and dispersed thoroughly with the aid of a mechanical stirrer (200 rpm) for 10 minutes. Coacervation was then induced by the addition of 100 ml of Petroleum ether slowly, over a period of 20 minutes, while stirring at the same speed. Stirring was continued for 15 minutes to rigidize the coating of microcapsules. The encapsulated product was then collected by vacuum filtration and air-dried to obtain discrete microcapsules. Microcapsules using five ratios of EC and DFS: 1:9 (ECMC-1), 1:4 (ECMC-2), 2:3 (ECMC-3), 3:2 (ECMC-4), and 3:1(ECMC-5) were prepared.

Characterization of Microcapsules

Drug Content: The accurately weighed microcapsules were taken into 100 ml volumetric flask; 5 ml of methanol was added and mixed thoroughly to dissolve the coat. To this 15 ml of the solvent blend consisting of one volume of Phosphate buffer of pH of 7.4 and four volumes of distilled water (this was used as dissolution medium in the present study and here after called 'dissolution medium') was added and the resulting solution was heated on water bath to evaporate the methanol. The solution was made up to volume with distilled water, suitably diluted and assayed at 276 nm.

Measurement of Microencapsulation Efficiency: 100 mg of the dried and loaded microcapsules were soaked in 100 ml of dissolution medium, until the concentration of released DFS reached a constant value. Then the microcapsules were separated and the concentration of DFS was determined by UV spectrophotometry at 276 nm. Then, Microencapsulation efficiency was calculated using the following formula:

Microencapsulation efficiency =
(estimated percentage drug content/theoretical percentage drug content) × 100. (1)

Yield: The percentage yield of microcapsules was calculated using the following formula:

Yield (%) =
(initial amount of raw materials/amount of microcapsules) × 100 (2)

The initial amount of raw material corresponds to the amount of drug plus polymer.

Evaluation of Dissolution of Diclofenac Sodium: Release of DFS from microcapsules (equivalent to 100 mg of medicament) and from tablets was studied using USP [10] Dissolution Type 2 apparatus. As mentioned earlier, a solvent blend consisting of one volume of Phosphate buffer of pH of 7.4 and four volumes of distilled water was used as dissolution medium. The stirring speed was set at 50 rpm and at 37 ± ?1° C.

Fitting of Dissolution Data: The kinetics of drug release from microcapsules was fitted to the following equations:

$Q = Q_0 + k_0 t$ Zero order kinetics equation (3)

$\ln Q = \ln Q_0 - k_1 t$ First order kinetics equation (4)

$Q = k_H t^{1/2}$ Higuchi equation (5)

Where, Q represents percentage of drug released at time t. In equations (3) and (4) k_0 and k_1 represent respective release rate constants. In Higuchi equation [11] (equation (5)) k_H stands for diffusion rate constant.

Results and Discussion

For microencapsulation with EC a non-aqueous phase separation method was employed. Toluene (solvent for EC) and Petroleum ether (non-solvent for EC) were used in optimum ratio of 1:2 for microcapsule formation [9]. Five ratios of EC and DFS, namely, 1:9 (ECMC-1), 1:4 (ECMC-2), 2:3 (ECMC-3), 3:2 (ECMC-4), and 3:1(ECMC-5) were used to prepare microcapsules. The microcapsules were white, free flowing and spherical in shape.

To characterize the microcapsules, three parameters were calculated: the drug content, the microencapsulation efficiency, and the weight yield. These parameters are helpful to ascertain whether the preparation procedure adopted for incorporating a drug into polymeric particles is efficient. Low s.d values in the mean percent drug content ensured uniformity of drug content in each batch of microcapsules. Also, microencapsulation efficiency and yield % are satisfactory (Table-1).

Table-1
Drug Content, Microencapsulation Efficiency, and % Yield of Microcapsules

Formulation	Drug Content (%)	Microencapsulation Efficiency	% Yield
ECMC-1	90.66(0.31)	100.73(0.45)	92.76
ECMC-2	79.00(0.43)	98.75(0.29)	89.96
ECMC-3	48.46(0.56)	80.77(0.23)	80.56
ECMC-4	35.45(0.65)	88.63(0.54)	72.59
ECMC-5	24.21(0.43)	96.84(0.46)	65.53

All values are averages of three determinations (n=3). Values in parentheses indicate standard deviation

DFS release from various microcapsules was found to be spread over varying periods of time (Fig.1). The drug release depended on the proportion of EC in microcapsules. Drug release decreased when the proportion of EC increased. However, drug release from microcapsules was very rapid,

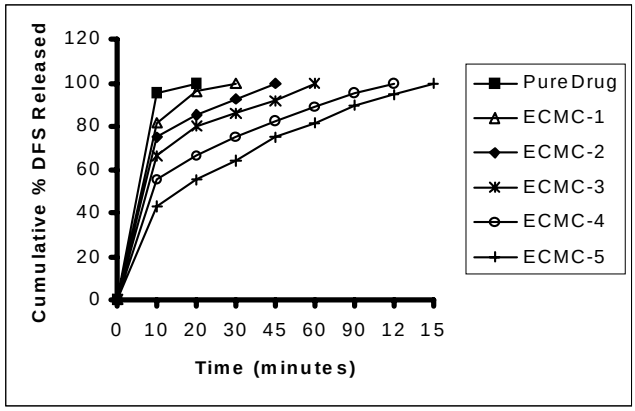

Fig.1. Dissolution profile of DFS from EC microcapsules

perhaps due to solubility of DFS in alkaline pH [9, 12]. The microcapsules didn't disintegrate during dissolution experiments, suggesting diffusion controlled process in drug release. There was a linear relationship between percent coat material (EC) in the microcapsules and T_{50} values(r=0.9078). The relationship could be expressed by the linear equation,

$$Y=5X+1.667 \quad (6)$$

Where, **X** is the percent coat material in the microcapsules and Y is the T_{50} values in minutes.

The dissolution data were fitted to various mathematical models (zero order, first order, and Higuchi's square root) to evaluate the kinetics of drug release from the microcapsules, using MS-Excel 2007 software. Coefficient of correlation (r) values were used to select the best fit for the data. Higuchi plots for various microcapsules are shown in Fig.2. The results are presented in Table-2, which indicate that drug release from microcapsules is diffusion controlled ($r \geq 0.9481$).

Table-2
Analysis of DFS Release Data of EC Microcapsules

Formulation	Coefficient of Correlation		
	Zero Order	First Order	Higuchi Equation
ECMC-1	0.8719	0.9943	0.9967
ECMC-2	0.8989	0.9932	0.9565
ECMC-3	0.9288	0.9982	0.9725
ECMC-4	0.9427	0.9875	0.9642
ECMC-5	0.9697	0.9793	0.9789

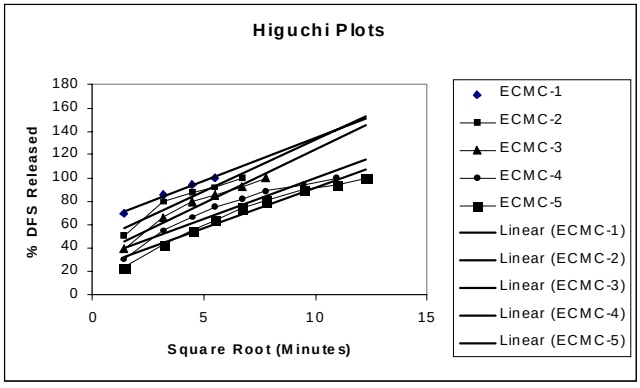

Fig.2 Higuchi Plots for Microcapsules

Conclusion

DFS could be microencapsulated by non-aqueous phase separation method using ethyl cellulose - toluene - petroleum ether system. The microcapsules were found to be discrete, loosely aggregated, and free flowing.

Percent of drug released from microcapsules was decreased with an increase in coat material in microcapsules. A linear relationship between percent of coat material in the microcapsules and the T_{50} values was observed.

EC can be used as rate controlling membrane and the non aqueous coacervation-phase separation technique employed is suitable for achieving Sustained Release.

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Chemical Composition and in Vitro Antimicrobial Activity of Essential Oils of Mentha Piperita

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ABSTRACT

The chemical composition of the essential oils obtained by hydrodistillation from fresh leaves of *Mentha piperita* cultivated in the south of Algeria (El-Oued) was determined by GC and GC/MS analysis. Among the 23 identified components menthol 51%, menthone 25%, 1,8 cineole 7% and menthyl acetate 6% were found to be the major ones.

Composition of essential oils extracted from leaves of *Mentha piperita* showed remarkable differences from the same species cultivated in other countries based on a comparison with published results. The in vitro antibacterial activity of the essential oils samples was tested on six strains, one yeast and one fungi. The test showed interesting antimicrobial properties especially on *Staphylo-coccus aureus* and *E.coli*.

KEY WORDS: essential oil, *Mentha piperita*, GC-MS, micro-organisms, antibacterial activity.

Introduction

In recent years much attention has been devoted to peppermint (*Mentha piperita L.*) as the result of its increased use in the pharmaceutical, cosmetic and food industries [1]. Since ecological conditions influence essential oil content and composition, there have been considerable number of papers discussing the most favorable growing conditions of peppermint [2-5]. Mint hybrids with good resistance to Puccinia menthae (mint rust), high yields of essential oil, and up to 70% of menthol, have been produced by crossing different mint varieties [6-8]. Essential oils which are a mixture of organic compounds are sometimes called plant secondary metabolites, a term which puts them in gategory of plant by products. They are obtained by physical process from odorous constituents located between the cells of the plants of thirty botanical families, with ninety species [9].The great importance of *Mentha piperita* essential oil is due to its germicidal bactericidal activities. Many medical properties have been referred to this oil, so it can be used in the treatment of several ailments. Mint is very profitable to the stomach, *Mentha piperita* oil, particularly as an inhalation, relieves nausea and respiratory problems and aids digestion. Digestive, carminative, respiratory, anti-inflammatory, balancing to be female system, cooling, clearing and muscle relaxant are peppermint oil properties [10].

The aim of this work is to extract and identify the main constituents of the essential oil obtained from leaves of *Mentha piperita* cultivated in the south of Algeria, and to compare their chemical composition with those extracted from the same species from different geographical origins, the work aims also to evaluate its antibacterial activity.

Materials and Methods

Vegetable Matter

The *Mentha piperita* family Labiatae was cultivated in the area of El-Oued, south of Algeria.

The Stocks

The stocks tested come from the laboratory of bacteriology of the medical sector of Biskra (Algeria) and from the medical analysis laboratory. They are human origins. The classification and the characteristics of the stocks tested were described previously [11].

Isolation of the Essential Oils

Fresh leaves of *Mentha piperita* were placed in the distillation flask of Clevenger apparatus, after 2.5 h of distillation, the volatile oil was collected dried over anhydrous sodium sulfate and stored at 20°C. The indices physico chemical were calculated according to standards' AFNOR [12].

Analysis of the Essential Oils

Analysis by FT-IR

IR spectra of essential oil components of the dried leaves were recorded on Testscan Shimadzu FTIR 8000 Spectrometer using liquid film. The FT-IR specter shows a large band at 3409.9 cm^{-1} corresponding to the alcohols function with 21% transmission, intense bands at 2925.8 and 2869.9 cm^{-1} corresponding to the C-H of alkanes, and intense band at 1710.7 cm^{-1}corresponding to the C=O function of ketones with 30% transmission.

Analysis by GC

The GC was carried out with Fisions instruments CHIMADZU: GC17A, with flame ionization detector using capillary column DB-5, 30mx0.25mm.The column temperature was programmed from 60°C to 240°C, at 3°C/min, using nitrogen as the carrier gas.

Analysis by GC-MS

The GC-MS was performed using instruments model AGILENT 6890 GC/CMSD 5973 equipped with a GL SCIENCE capillary column HP5MS (30m, 0.25mmID; 0.25µm film thickness) and a 70 eV EI Quadruapol detector. For GC¨CMS detection, an electron ionization system with ionization energy of 70 eV was used.

Helium was the carrier gas, at a flow rate of 1 ml/min. Injector and MS transfer line temperatures were set at 250 and 220 °C, respectively. Column temperature was initially at 60 °C held for 2 min, then gradually increased to 125 °C at a 2 °C/min rate, held for 2 min, and finally increased to 220 °C at 5 °C/min held for 2 min. Diluted samples (1:100 v/v, in ethanol) of 1.0 ml were injected manually using splitless mode.

Identification of Essential Oil Constituents

The components of the essential oils were identified by comparing their retention indices and mass spectra fragmentation patterns with those stored on Wiley 275 MS computer library built up using pure substances or with authentic compounds and confirmed by comparison of their retention indices.

Antimicrobial Test

The inhibiting activity of the E. oil towards the bacteria was tested by the following two methods:

Disc method

The culture medium used, in this disc method, for all assays seeded Iso-sensitest Agar (marketed by Pasteur institute Algiers).

Minimal Inhibitory Concentration (MIC) method

The Petri plate (85mm) are put to incubate with 37 °C after having inoculated the micro-organism on iso-sentitest Agar, the essential oil is then added, the minimal concentration which inhibits the growth of the bacteria is then determined. With regard to fungi it was tested only by the method of MIC and that is carried out using the discs of 4mm of diameter cut out starting from the Agar (sabauraud) supporting the culture of a mushroom (7 days old).

Results and Discussion

Essential oil yields of the plant indicated in Table 1, the yields ranged from 0.92 to 3.7 % depending on the method of extraction. The greatest yield was that obtained by hydro-distillation (3.7%).

Physical and chemical characteristics (specific gravity, refractive index, optical rotation, ester indices, acid indices and solubility in ethanol 70%) of essential oil of *Mentha piperita* compared to those reported in literature are given in table 2.

Table 1
Extraction yield of essential oil

Vegetable matter	Essential oil(g)[a]	yield (%)[b]	yield (%)[c]	yield (%)[13]
Mentha piperita	3.7	0.92	2.7	2.2

[a] (g)of E. oil obtained by hydro distillation of three extractions, [b]yield in % of E oil obtained starting from the extracted fresh matter, [c]yield in % of HE obtained starting from 200g extracted dry matter.

Table 2
Physical and chemical properties of *Mentha piperita* oil

E.oil	D^{20}_{20}	η^{20}_{D}	α^{20}	IA	IE	Solubility at 70%
Mentha piperita	0,900	1,4633	-14	0.57	17	not necessary used plus of 4V (70%)
Mentha piperita	0.901	1.4600	-29	–	14	5v
(France) [14]	0.916	1.4670	-10		19	

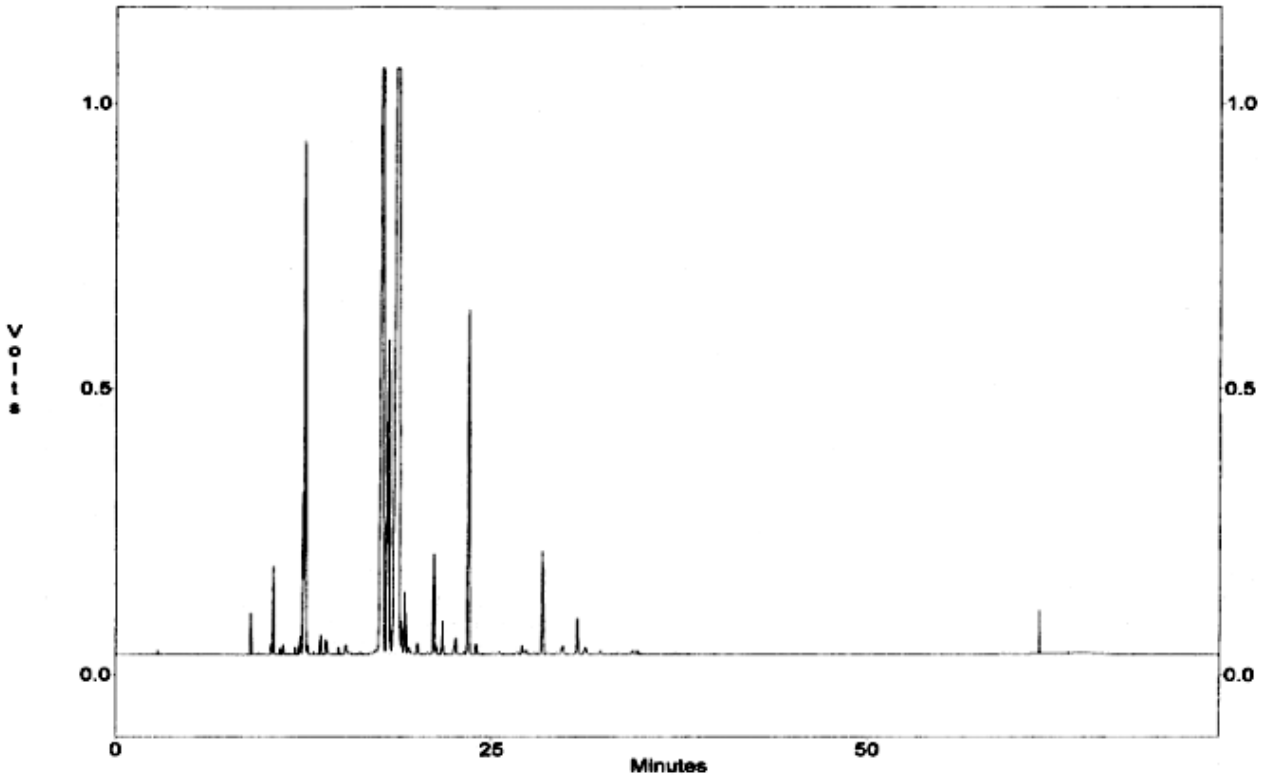

Fig.1: GC Essential oils chromatogram of *Mentha piperita* leaves

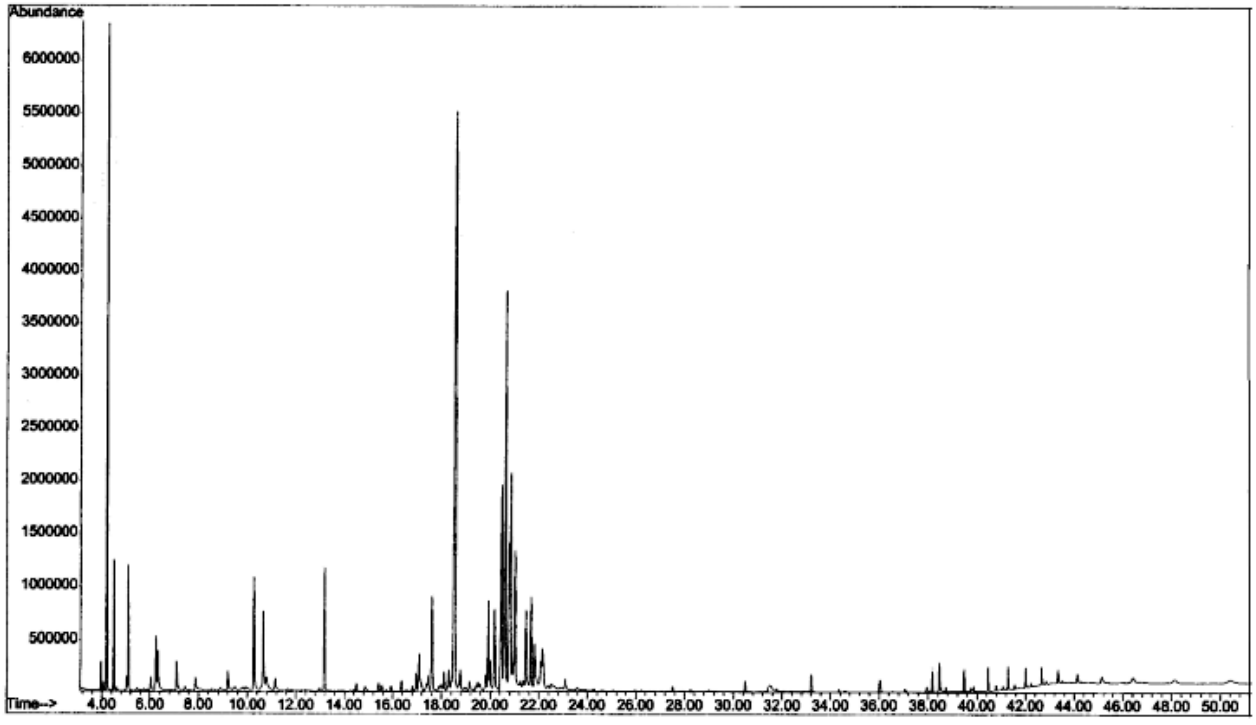

Fig.1: GC-MS eessential oil chromatogram of *Mentha piperita* leaves

GC and GC-MS Analysis

Compounds were identified by comparison of their retention indices with those reported in the literature [15]. The retention indices (I_p) were calculated according to the relation of Ven Dan Dool and Kratz [16] under TPGC conditions at a linear programming relative to C-8 to C -25 n-alkanes.

Table 4:

Chemical composition of *Mentha piperita* oil identified by GC

Trs	Ip	Compound name	Ip[17]
8.958	936	α- Pinene	935
10.475	979	β- Pinene	978
12.458	1030	limonene	1031
12.617	1034	1,8 cineole	1036
17.850	1160	menthone	-
18.158	1168	Neomenthol	-
18.900	1185	Menthol	-
19.250	1193	Neoisomenthol	1193
21.183	1239	Pulegone	-
21.758	1252	Piperitone	-
23.483	1293	Menthyl acetate	-
28.375	1417	β- caryophyllene	1418
30.683	1478	β- selinene	-

The analysis of GC-MS confirmed and infirmed the compounds proposed by the GC analysis. The results showed that of total volatile compounds identified in *Mentha piperita* essential oil were monoterpinoids which include: menthone, pulegone, menthol, piperitone, menthyl acetate and 1,8-cinéole. Three monoterpenes were identified: α-Pinene , β- Pinene and limonene. Three sesquiterpenes were identified as a minor compounds; β- bourbonene, β-selinene, germacrene-D. Globulol, caryophellene oxide and spathulenol were identified as oxygenated sesquiterpene compounds. One non-terpene volatile compound was identified ; 3- Octanol.

The major component was menthol 51%, menthone 25%, 1,8 cineole 7% and menthyl acetate 6%.

The concentration of the essential oil components can be laid down in three categories, i.e. trace (<1%), minor (<10%) and major (>10%) components. Compounds identified by GC-MS are regrouped in table 5.

The obtained results indicate that monoterpenoids, menthoe, menthol, menthyl acetate and 1,8-cineole are characteristic volatile compounds of *Mentha piperita* essential oil . These results for qualitative and quantitative analysis of *Mentha piperita* oil are in agreement with those reported by Piccaglia and Marotti [18].

Table 5:

Essential oils composition (% w/w) identified by GC-MS of leaves of *Mentha piperita* cultivated in south of Algeria.

Tr	Compound name	Percentage
3.96	α- Pinene	t
4.49	3- Octanol	t
6.04	β- Pinene	t
6.23	limonene1.6	
7.10	1,8 cineole	7.15
9.20	Globulol0.2	
10.27	menthone	25.34
10.65	Neomenthol	t
13.18	Menthol51.17	
16.97	Neoisomenthol	t
17.09	Pulegone	t
17.46	Piperitone	t
18.12	Menthyl acetate	5.9
18.55	Neomenthyl acetate	t
19.83	β- Bourbonene	0.42
20.00	β- caryophyllene	t
20.18	D Germacrene	0.50
20.45	β- selinene	0.20
20.83	Spathulenol	t
21.1	Caryophyllene oxide	0.30

T_r retention time
t = trace (< 0.05%)

Antimicrobial Activity

Antibacterial Activity

Two methods are used, discs diffusion and inhibiting minimal concentration determination methods. The essential oil of *Mentha piperita* at two different concentrations (2 and 3µl) was tested against some micro-organisms. The inhibition zone diameter in produced by the application of the essential oil on various micro-organisms is shown in table 4 and table 6.

The essential oil of *Mentha piperita* was active against all of the tested bacteria with inhibition zone diameter at 2 µl of essential oil as follows: *S.a* 15 mm, *E.c* 12 mm, *S.b*17 mm, *S* .α 20 mm, *P.m* 13 mm and *P. a* 16 mm. At the same concentration the inhibition zone was 14 mm about the yeast *L.c.a.*

These results indicate that the essential oil was active for tested bacteria and yeast, the Gram positive is more sensible than yeast and Gram negative bacteria. The sequence of sensibility can be arranged as follows:

Gram positive > yeast > Gram negative

The MIC confirmed the results obtained previously; Gram negative bacteria was resistant, for example the MIC of bacteria *P.a* is 15 $mg.ml^{-1}$ and 12 $mg.ml^{-1}$ for the yeast *Lca*, and 6 $mg.ml^{-1}$ for *S.a.*

Antifungal Activity

The inhibition zones diameter of fungi by the essential oil are shown in table 7.The results showed that *Mentha piperita* oil was effective on fungi.

* the control culture has a mycelial growth until the third day and arrives almost in edge of Petri plates (45mm) dice the seven days.

* For a concentration of 30 $mg.ml^{-1}$ *Mentha piperita* oil of 80% of the fungic growth is inhibited by the *Mentha piperita* oil.

The antimicrobial effects of the oil of *Mentha piperita* species against several types bacteria, yeast and fungi is due to their constituents of menthol and menthone, this have been revealed by investigations of numerous authors [13,19].

Conclusion

Essential oils extracted by hydro-distillation from the leaves of *Mentha piperita* growing in the south of Algeria (El-Oued) were found to be rich in menthol, menthone, 1,8-cineole and menthyl acetate. Composition of the Algerian essential oils showed remarkable differences from the same species cultivated in other countries. These differences could be due to climatic conditions. The inhibitory effect of the essential oil of this plant tested on some strains, yeast and fungi may due to its high content of menthol. The study showed interesting antimicrobial properties especially for the pathogene strains as *S. aureus* and *E.coli.*

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Table 6

Antimicrobial activity from leaves essential oil of *Mentha piperita* cultivated in Algeria.

Bacteria mg/discs		Gram - Positive			Gram - Negative			L c a
		S.b	S.a	S.α	E.c	P.m	P.a	S.b
Mentha.p	2	17	15	20	12	13	16	14
	3	22	19	25	13	15	19	

S.b : Staphylococcus blanc **S.a** : Staphylococcus aureus **E.c** : Escherichia coli **S.α** : Streptocoque α, **P.a** : pseudomonas aeurogenosa Hémolytique **L c.a** : Levure candida albicans, **P.m** : Proteus mirabilis.

Table 7

Measure fungic growth of mushroom

Number of days mg/ml		0 day	1st day	2nd day	3rd day	4th day	% of inhibition [20]
Control		4 mm	20	24	28	43*	0%
Mentha.p	4	4 mm	19	22	25	35	20.51%
	30	4 mm	4	10	12	13	76.92%

***** the colonies arrive in edge of limps of Petri (45 mm) the values are given in mm and corresponding to the fungi growth (including the diameter of the disc of fungi 4 mm).

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