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Review

Instabilities of proteins: theoretical aspects, degradation products and methods for their detection

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Abstract

Recombinant DNA technology has led to a significant increase in the number of peptide and protein based pharmaceuticals, giving a new approach to combat poorly controlled diseases. This particular development has been reached in the last two decades. However, proteins are highly susceptible of physical and chemical degradation resulting in a decrease or complete loss of biological activities. Reasons for their physical and chemical instabilities and the methods for their examination, become a challenge for the pharmaceutical scientists for successful development of stabile protein - based pharmaceuticals.

The stability of protein - based pharmaceuticals is significant in terms of their pharmaceutical quality and biological activity. In addition, a right choice of suitable analytical methods is needed in order to detect an early formation of degradation products or modified forms.

Key words: proteins, physical instability, chemical instability, methods

Inroduction

During the past two decades, peptides and proteins have become an important class of potent therapeutic drugs. They have gained significant importance in the treatment of several severe diseases including autoimmune diseases, memory impairment, hormonal disorders, organ transplantations and different cancers. Major advantages of protein drugs are both their extremely specific activity and their high tolerability (1).

While recombinant DNA technology, which is providing exciting opportunities for new pharmaceutical development and new approaches to the diagnosis, treatment and prevention of diseases, has led to a significant increase in the production of peptides and proteins for pharmaceutical purposes, this has not been matched by the number of peptide and protein based drugs available on the market. The reasons for that are multiple: proteins highly susceptibility to chemical and physical degradation, which is associated with relevant difficulties in purification, storage and delivery, multifaceted metabolic properties, variable tissue penetration and toxicity related to the stimulation of the immune or allergic reaction (2, 3, 4).

The structure and function of proteins is determined by their amino acid sequence which defines the peptide backbone, *the primary structure*. In spite of the enormous number of naturally occurring proteins, a mere of 20 amino acids construct proteins. The vast difference in the three-dimensional structure and, therefore, also in protein function originates solely from a different amino acid sequence, i.e. the unique structure of a protein is determined by the chemical and physical properties of the amino acids aligned within the protein sequence. *The secondary structure* describes the folding or the shape of the polypeptide chains into regular, ordered structures like α-helices and β-sheets. Furthermore, areas with increased flexibility – the so-called turns or loops – are to be subsumed in this level of protein structure. The domains of the secondary structure and all noncovalent interactions such as hydrogen bonds and hydrophobic, electrostatic, or van der Waals interactions generate the intrinsic, three-dimensional arrangement of a protein, *the tertiary structure*. Some proteins consist of several

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polypeptide chains. *The quaternary structure* characterises the non-covalent interactions binding these chains into a single protein molecule (5). For example, haemoglobin consists of four polypeptide chains, which are associated by one Fe^{2+} ion. The retention of the tertiary structure is deemed the primary requirement for the biological activity of protein molecules. However, the biochemical and structural complexity of these molecules is the reason for proteins to react sensitive to even marginal changes in their natural environment (6).

In the context of protein structure, the term stability can be defined as the tendency to maintain a native (biologically active) conformation.

As proteins and peptides continue to enter the pharmaceutical market, their stability becomes a pressing issue for the pharmaceutical scientists. Native proteins are only marginally stable and highly susceptible to degradation, both chemical and physical (7-9).

Chemical instability refers to the formation or destruction of covalent bonds, within a protein molecule, i.e. some amino acid side chains are chemically reactive, whereas others are chemically inert. It has been demonstrated that „labile" amino acid residues are susceptible to covalent modifications via bond formation or cleavage through nonenzymatic reactions, including hydrolysis, deamidation, oxidation, racemisation, β-elimination and cystine destruction/disulfide exchange (10, 11). These changes alter the primary structure of the protein, and impact higher level of its structure.

Physical instabilities include aggregation and precipitation, and adsorption to surface (7- 9, 12,). Chemical instabilities such as deamidation and disulphide bond cleavage, may also lead to physical instabilities, and vice versa (6, 11).

The primary focus of this minireview is to present and discuss the main reasons for the most common physical and chemical instabilities and the methods for their detection. It is important to remember that every protein is unique, both physically and chemically, and therefore exhibits unique stability behaviour.

Denaturation

The loss of tertiary structure, and frequently also of secondary structure is generally referred to as denaturation of the protein. Perturbation of secondary or tertiary structure can lead to exposure of previously buried amino acid, facilitating its chemical reactivity; thereby leading to loss of its native or original characteristics. Denaturation can be caused by destabilizing agents such as excipients (reducing sugars, antioxidants, surfactants, metal ions), heat, hydrolysis by strong acid or alkali, enzymatic action, exposure to urea or other substances, or exposure to ultra violet light. Excipients like reducing sugars can react with protein amino groups to form schiff's bases (Maillard reaction) which can be shown by LC/MS (13). Antioxidants may contain reducing agents that will destroy disulphide bonds. Several surfactants like Tween 20 and Tween 80 can cause oxidation of aminoacid due to residual peroxides present in these materials, which can be detected by RP-HPLC (14). Many chemical reactions involving polypeptides and proteins are catalyzed by metal ions such as Zn^{2+}, Cd^{2+}, Pb^{2+}. At high temperature (80-100°), aspargine and glutamine are susceptible to deamidation, Aspartate-Xaa peptide bonds are susceptible to hydrolysis, disulphide bonds rupture, and Xaa-Prolin peptide bonds undergo cis-trans isomerisation (where Xaa is any amino acid). High temperature can result in physical degradation due to irreversible denaturation. Residual moisture can be responsible for protein instability in the solid state. pH has also strong influence on denaturation and aggregation rate (15, 16).

The folded state of proteins is connected to conformational stability, which is expressed as the free energy change ΔG during the unfolding/denaturation reaction under physiological conditions (17). The higher the ΔG value, the greater the stability of the protein. However, the reported ΔG values for proteins of 45 +/- 15 kJ/mol indicate that the folding state is only marginally more stable than the denatured state (18). As a matter of fact, the conformational stability of a protein in aqueous solution tallies with only a few hydrogen bonds or ion pairs (2). Simplified, protein denaturation can be described as:

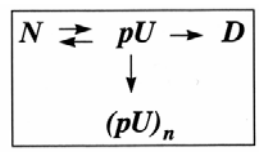

Figure 1: Equation of the native and denatured state of a protein

The native state N exists in an equilibrium with a partially unfolded state pU. This unfolding of the native protein can be reversible, e.g. an increase of the temperature causes unfolding, which can be reversed by a subsequent temperature decrease (7). Generally, the loss of the tertiary structure implies an increase in the protein molecule's reactivity, which results in a decrease in their stability. Hydrophobic regions, which were accumulated in the core of the folded protein, are then exposed to surrounding solvents. As a consequence, side reactions can now lead to an irreversible, denatured state D. Alternatively, partially unfolded proteins may encounter irreversible aggregation (pU)n. Constantly elevated temperatures, extreme pHs, the formation of interfaces during shaking, shearing, adsorption to hydrophobic surfaces, high pressure, and denatu-

rants such as urea and guanidine hydrochloride foster irreversible transitions (2,19-23).

Physical instability

Physical instability of proteins denotes that these super structured molecules can undergo changes independent of any chemical modification, i.e. includes the reactions that do not involve the formation or cleavage of covalent bonds.

Aggregation and precipitation

Protein aggregation is arguably the most common and troubling manifestation of protein instability, encountered in almost all stages of protein drug development, during refolding, purification, sterilization, shipping and storage processes (24).

The aggregation of protein molecules into non-native assemblies in vivo can have profound pathological implications, as in the aggregation of β-amyloid proteins in Alzheimer's disease and the aggregation of prion protein in numerous neurodegenerative diseases (24-26). The presence of aggregates in therapeutic protein pharmaceuticals can cause adverse effects within patients, ranging from immune response to anaphylactic shock (27, 28).

Protein aggregation is defined as the association of at least two denatured protein molecules. With increasing numbers of molecules the solubility of these species will decrease, eventually resulting in the precipitation of the protein. Aggregation can occur even under conditions where the proteins native conformation is favored thermodynamically compared to the unfolded state and at concentrations well below the proteins solubility limit (24, 29, 30). To effectively inhibit aggregation, both in vivo and in vitro, a more complete understanding of the mechanisms by which proteins aggregate is needed (31).

Mechanisms of protein aggregation are still not fully understood. One plausible mechanism is that aggregation is catalyzed by the presence of a small amount of a contaminant. That contaminant could be a damaged form of the protein product itself, host cell proteins, or even non-protein materials such as silica particles (32). Damaged forms of a protein product can arise from chemical modification (such as oxidation or deamidation) and from conformationally damaged forms arising from thermal stress, shear, or surface-induced denaturation. A second mechanism begins with partial unfolding of the native protein during storage. Protein conformation is not rigid – the structure fluctuates around the time-averaged native structure to different extents depending on environmental conditions. Some partially or fully unfolded protein molecules are always present at equilibrium in all protein solutions, but most such molecules simply refold to their native structure. However, those unfolded proteins may instead aggregate with other such molecules or may be incorporated into an existing aggregate nucleus, eventually to form larger aggregates as described above. Factors such as elevated temperature, shaking (shear and air-liquid interface stress), surface adsorption, and other physical or chemical stresses may facilitate partial unfolding. A third aggregation mechanism is reversible self-association of the native protein to form oligomers. The tendency of different proteins to reversibly associate is highly variable, and the strength of that association typically varies significantly with solvent conditions such as pH and ionic strength. Such reversible oligomers often eventually become irreversible (they are a first step along a pathway to irreversible aggregation). Detection of reversible aggregates can be especially challenging. One of the reasons is that such aggregates can dissociate from dilution during the measurement process (33-36).

Regardless of the mechanism of aggregation, preventing aggregation problems requires sensitive and reliable technologies for quantitative determination of aggregate content and aggregate characteristics (37). Size-exclusion chromatography (SEC) has been a workhorse for detecting and quantifying protein aggregation (2, 38-39). Native gel electrophoresis and SDS-PAGE have also been used to observe protein aggregation. Column-free techniques such as analytical ultracentrifugation (AUC), field-flow fractionation (FFF), and dynamic light scattering (DLS) now find increasing application in aggregation analysis (36-37, 40). As it can be concluded, there are various techniques for assessing protein aggregation, which will enable detection of dimmer and aggregate formation at the very beginning. All that is important for pharmaceutical quality and biological activity of protein - based drugs.

Chemical instability

Chemical instability of proteins can be potentiated or induced by temperature, pH, light and composition of the formulation buffer. Such chemical modifications may result in alteration of structure, loss of function, acquired immunogenicity and altered pharmacokinetics.

Oxidation

Methionine, cystine, tryptophan and tyrosine residues are susceptible to oxidation. Air, residual peroxide content, or intense fluorescent light, can convert thioether to sulfoxide, and then sulfone (41-46). The polysorbate-80

and polysorbate-20, which are usually used in the protein formulations like surfactants, have a tendency to produce peroxides, which in turn, can lead to oxidation of the methionine residues. This peroxide contamination appears to have a greater effect on protein oxidation than the presence of atmospheric oxygen in the vial headspace or the effects of product foaming in the fill lines (14). Oxidative modification can be variable (47). Peptide maps are convenient for detecting methionine oxidation, and RP-HPLC is used to separate the oxidized forms. Oxidation causes a protein to become more hydrophilic and oxidized proteins elute before the native form in RP-HPLC (48).

Oxidation occurs in a number of proteins favored by factors like temperature, pH etc. Most proteins lose biological activity when oxidized. Thereby, formulation approaches include addition of anti oxidants (sodium thiosulphate, catalase, or platinum) and adjustment of environmental conditions (pH or temperature) to prevent oxidation (49).

Deamidation

For many proteins deamidation is one of the most often observed stability problems and occurs more rapidly than any of the other degradation routes (14). Deamidation of asparginine residues (glutamine residues to lesser extent) to aspartate or isoaspartate via succinimide intermediates (positive to negative charge) is a major cause of spontaneous degradation and loss of aminoacid sequence homogeneity. Deamidation can make protein prone to proteases and denaturation. This can affect the *in vivo* half-life, activity, and conformation of protein, and also increase the immunogenicity of certain protein. Deamidation often results in the loss of biological activity (50, 51).

The deamidation process is mainly dependent on the storage temperature, on the pH of the formulation buffer and on the sequence and conformation. Deamidation can be detected by isoelectric focussing and quantified by densitometric scanning of the gel and by RP-HPLC (50, 52, 53). A common means of determining deamidation is to digest the protein with tripsin and to look for new peptide fragments eluting slightly later than fragments containing asparagine in RP-HPLC, because under acidic conditions aspartic acid is slightly more hydrophobic than asparagines (50). At neutral pH, fragments containing asparagines elute after the aspartic acid deamidation products because they are less hydrophobic under these conditions (53).

Conclusions

Proteins are only marginally stable and highly susceptible to physical and chemical degradation. Physical and chemical instabilities of proteins are the major road barriers, hindering rapid commercialization of potential protein drug candidates. Thereby, the successful development of stabile protein-based pharmaceuticals is highly dependent on a thorough understanding of their physico-chemical and biological characteristics and of the mechanisms by which proteins stabilizes.

Due to the complexity and heterogeneity of protein structures, multiple analytical methods for stability testing must be employed. Stability indicating test methods must be validated to be suitable to detect potential degradation products or modified forms. Detection of degradation products or modified forms at the very beginning signalling the stability changes important for pharmaceutical quality and biological activity of protein - based pharmaceuticals.

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Резиме

Нестабилност на протеини: Теоретски аспекти, деградациони продукти и методи за нивно детектирање

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Клучни зборови: протеини, физичка стабилност, хемиска стабилност, методи

Рекомбинантната ДНК технологија доведе до значителен пораст на бројот на пептидни и протеински фармацевтски препарати, давајќи нов пристап во третманот на оние заболувања, за кои досега применуваните фармацевтски препарати не даваат задоволителни резултати. Особено брз развој на ова поле е постигнат во последните две децении. Меѓутоа, како проблем се јавува нестабилноста на протеинските молекули. Имено, познато е дека протеините се подложни на физичка и хемиска деградација, што резултира со намалување или целосно губење на нивната биолошка активност. Токму поради тоа, причините што доведуваат до нивна физичка и хемиска нестабилност, како и методите за нивно испитување, претставуваат предизвик за фармацевтите за развој на стабилни протеински фармацевтски препарати.

Стабилноста на протеинските фармацевтски препарати е многу значајна во однос на нивниот фармацевтски квалитет и биолошка активност. Поради тоа, потребен е правилен избор на соодветни аналитички методи кои ќе овозможат детектирање во почетниот стадиум на формирањето на дегдрадационите продукти или модифицирани форми.

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Original scientific paper

Evaluation of urinary proteins in healty full-term neonates by SDS-PAGE

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Abstract

The pattern of urinary proteins in healthy full-term neonates was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), coupled with determination of few parameters related to urinary protein excretion.

Twenty healthy full-term neonates were included in the investigation. Five urine samples from each subject were collected on days 3, 7, 14, 21 and 28 after birth. Determination of total proteins was performed using turbidimetric method with sulfosalicylic acid. Urinary creatinine concentration was determined by Jaffe method. Urinary proteins were separated by horizontal gradient SDS-PAGE according to Görg.

The highest values for total urinary proteins and for protein/creatinine ratio were detected in urine samples excreted on days 3 and 7 after birth. Three types of SDS-PAG electrophoretic profiles were observed. The first type of electrophoretic profile was characterized by the presence of proteins of mixed glomerular and tubular origin with molecular weights from 10 to 160 kDa. Typical for the second type of electrophoretic profile was the presence of two faint fractions with molecular weights of 78 and 90 kDa and several intensive low molecular weight fractions (14-67 kDa). In the third type of electrophoretic profile only low molecular weight proteins (10-67 kDa) were detected in all five urine samples. These findings express the transitory immaturity of the glomerular filter and tubular protein reabsorbing system of the newborn kidney. Apparently, the tubular protein handling normalizes later than the glomerular filtration of proteins.

Key words: Proteinuria; neonates; SDS-PAGE; electrophoretic profiles

Introduction

It has been clearly demonstrated that the renal cells are not fully differentiated at birth and that many of the differences in renal function seen between infants and adults should be attributed to immaturity (1). This renal immaturity does not seem to carry any risk in healthy full-term infants fed an appropriate diet, but can represent a major risk in situation of disease, inappropriate fluid and electrolyte losses, and exogenous pharmacologic stress (2).

Renal insufficiency and renal failure of newborns in an intensive care unit is a very common problem. It was reported that between 8 and 24 % of neonates in special care wards experience renal failure (3). Acute renal failure is a recognized complication of birth asphyxia and may result in permanent renal damage in up to 40 % of survivors (4).

Taking all these facts into account it is obvious that evaluation of renal glomerular and tubular functional and structural integrity in neonates is of great importance. For this purpose in clinical practice the investigation of a battery of biochemical parameters is frequently undertaken.

Measuring of serum concentration of creatinine, as well as cistatin C is frequently used as a parameters for evaluation of glomerular function (5). A more precise assessment of the functional capacity of the kidney is made by measuring glomerular filtration rate (GFR), but this procedure is expensive, time consuming and invasive because puncture of peripheral vein is necessary to obtain several blood samples (6,7).

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It is generally recognized that urine, as a medium**,** is a source of great number of diagnostic information. Determination of concentration of total urinary proteins, as well as measuring of concentration of different urinary proteins has been used to monitor kidney and urinary tract disease for more than 150 years. The determination of total urinary protein does not differentiate between glomerular and tubular proteinuria. It has been demonstrated that different urinary proteins carry different diagnostic information (8). So, urinary concentration of high-molecular proteins, especially albumin and immunoglobulin G, are the best markers for assessment of glomerular function. Determination of urinary concentration of low-molecular weight proteins, such as α_1-microglobulin, β_2-microglobulin and retinol binding protein (RBP) is recommended as potential markers for evaluating function of renal proximal tubules (9). Measuring of fractionary excretion of urinary sodium is used for investigation the functional reabsorption capacity of the distal segment of the nephron. Of all urinary enzymes, the brush-border membrane enzyme leucine-aminopeptidase (LAP) and the lysosomal N-acethyl-β-D-glucosaminidase (NAG) were recommended as markers for investigation of the structural integrity of renal proximal tubules (10).

In the recent years the accent is put on separation of urinary proteins using high resolution electrophoretic techniques, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), capillary electrophoresis and 2D-electrophoresis (11).

The results from great number of studies have shown that SDS-PAGE is a method of choice for detection of early renal lesions (12). It is also very attractive method, because of its non-invasive nature, relatively low price and accuracy.

In the present work the patterns of urinary proteins in healthy full-term infants during the first month of life were investigated by SDS-PAGE, coupled with determination of few parameters related to urinary protein excretion.

The purpose of this examination is to evaluate SDS-PAGE profiles characteristic for healthy newborn infants; this is the basis for further evaluation of SDS-PAGE in assessment of glomerular and tubular function in conditions in which renal function might be affected (such as hypoxic ischemic encephalopathy, sepsis, nephrotoxic agents etc.).

Experimental

The present investigation was conducted on 20 healthy full-term neonates with gestational ages ranging from 36 to 41 weeks (mean 38.2 ± 1.10 weeks).

Urinary samples excreted on day 3,7,14,21 and 28 after birth were used for all examinations. Collection of urine samples was made by using a collection bag placed over the infants' external genitals. All samples were taken promptly to the laboratory and centrifuged on 3000 rpm for 15 minutes.

Total urinary proteins were determined by the Meuleman`s method, using sulfosalicylic acid (13). Urinary creatinine concentration was determined by Jaffe method (14). Proteine/creatinine ratio (P/C) was calculated by dividing the total urinary protein concentration with urinary concentration of creatinine.

Separation of urinary proteins was performed by horizontal ultrathin pore gradient-dodecyl sulfate electrophoresis according to Görg et al. (15). Urinary samples were neither diluted nor concentrated; 0.9 ml urine with 0.1 ml sample buffer (1.5 M Tris/HCl, 10% SDS, pH 8.8) was incubated for 3 minutes in boiling water. SDS-polyacrylamide gradient gels (4-22 %) were prepared by standard procedure in dimensions 195 x 250 x 0.5 mm. SDS-PAGE worked at 5^{o}C for 2 hours on Multiphor II Unit, LKB (Brown, Sweden). Pharmacia LKB low-molecular weight calibration proteins were used for determination of molecular mass of separated protein fractions. Gels were stained with Coomassie Blue R-250 and prepared in stable preparations.

Results and discussion

Results regarding the concentration of total urinary proteins and P/C ratio in healthy full-term neonates on days 3,7,14,21 and 28 of life are presented in Table 1.

The highest values for total urinary proteins in healthy full-term neonates were detected in urinary samples excreted on days 3 and 7 after birth with upper value of 405 mg/L and lowest values in samples excreted on days 21 and 28 of life with range between 10-50 mg/L.

The highest detected value for P/C ratio was detected in urinary samples excreted on day 3 of life (480 mg/g cre-

Table 1. Data (arithmetic mean and range) for total urinary proteins and P/C ratio on days 3,7,14,21 and 28 after birth in healthy full-term neonates (n=20)

	Healthy full-term neonates				
Parameters	Day 3	Day 7	Day 14	Day 21	Day 28
Total urinary proteins	156	130	32	29	25
(mg/L)	(10 - 405)	(10 - 355)	(10 – 120)	(10 – 50)	(10 – 50)
P/C ratio	190	297	139	153	134
(mg/g Creatinine)	(30– 480)	(38 – 460)	(30 – 415)	(80–248)	(36 – 369)

atinine). The highest mean arithmetic value for P/C ratio was detected in urine samples excreted on day 7 after birth (297 mg/g creatinine). The differences in arithmetic means for P/C ratio between day 3 and day 28 were not so expressive like for the total urinary proteins concentration.

Fig.1, 2 and 3 present typical SDS-PAGE profiles obtained from separation of urinary proteins in neonates, included in conducted investigation. For electrophoretic separation of each subject, five different urinary samples (excreted on day 3,7,14,21 and 28 of life), with laterally applicated protein markers, were used.

In 40 % of neonates (n=8) electrophoretic profiles were characterized by the presence of proteins of mixed glomerular and tubular origin, detected in all five urinary samples. The molecular weight of proteins were found to be distributed over a wide range from 10 to 160 kDa. The intensity of fractions was highest in urine sample excreted on days 3 and 7 and decreased thereafter. On day 3 the fractions with highest intensity were those with molecular weight of 67 kDa (albumin) and molecular weight of about 30 kDa. One week after birth the low molecular weight proteins predominated because there was a substantial decrease in the excretion of albumin and of high molecular weight proteins (Fig. 1).

In 20 % of neonates electrophoretic profiles were characterized with presence of two faint fractions with molecular weights of about 78 kDa and 90 kDa, and several intensive and higher proportional low molecular weight fractions (from 14 to 67 kDa). Fractions with molecular weight of about 45 kDa, 30 kDa and 14 kDa were the most prominent in urinary samples excreted on days 3 and 7 after birth. The intensity of all fractions decreased in urinary samples excreted on days 14, 21 and 28 after birth (Fig. 2).

In other 40% (n=8) of newborns in all urinary samples only the presence of low molecular weight proteins (from 10-67 kDa) was detected. The intensity of fractions was highest in urinary samples excreted on days 3 and 7 after birth and lowest in samples excreted on days 21 and 28 of life. In 7 of neonates with this type of electrophoretic profile in samples excreted 21 and 28 days after birth, the presence of only albumin fraction was detected (Fig. 3).

Physiological proteinuria in adults is well known with many studies regarding the determination of physiological excretion of different proteins as well as separation of urinary proteins with SDS-PAGE (16,17,18). There are also a great number of studies related to quantitative changes in some specific urinary proteins during the first month of life and later in early infancy and older children (19, 20). There are only a few reports in literature regarding the SDS-PAGE electrophoretic profiles in healthy newborns (21-23).

In view of the lack of knowledge about physiological proteinuria in neonates, the present study was undertaken to

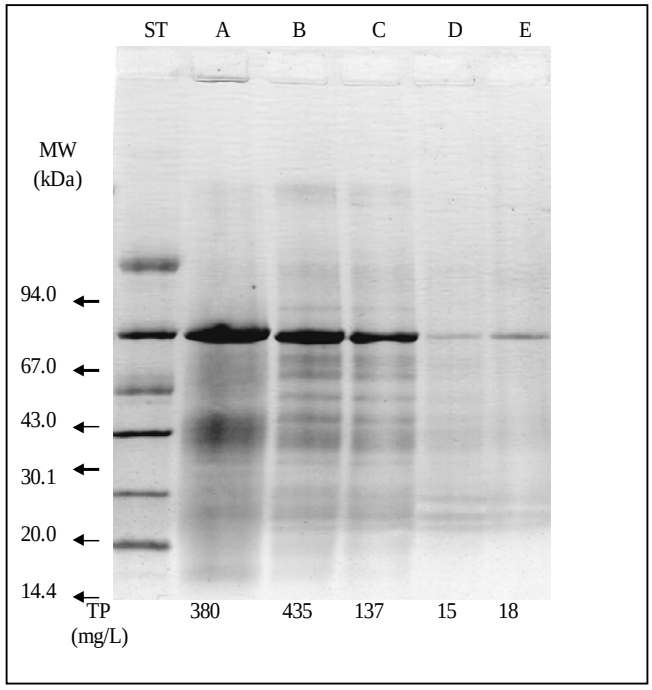

Fig. 1 Electrophoretic profiles of urinary proteins in healthy full-term neonate 3, 7, 14, 21 and 28 days after birth (A,B,C,D,E)
TP: total urinary proteins in mg/L, MW: molecular weight, ST: Standard (Pharmacia LKB LMW calibration proteins)

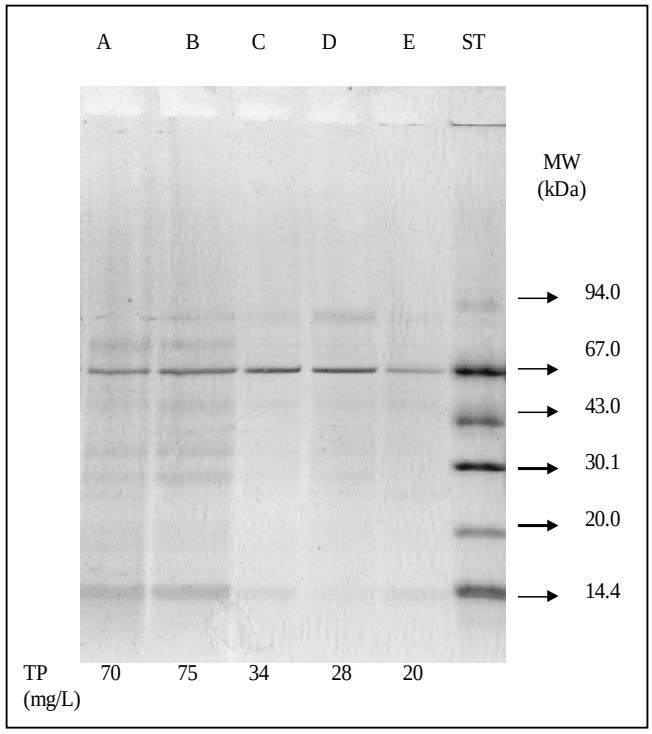

Fig. 2 Electrophoretic profiles of urinary proteins in healthy full-term neonate 3, 7, 14, 21 and 28 days after birth (A,B,C,D,E)
TP: total urinary proteins in mg/L, MW: molecular weight, ST: Standard (Pharmacia LKB LMW calibration proteins)

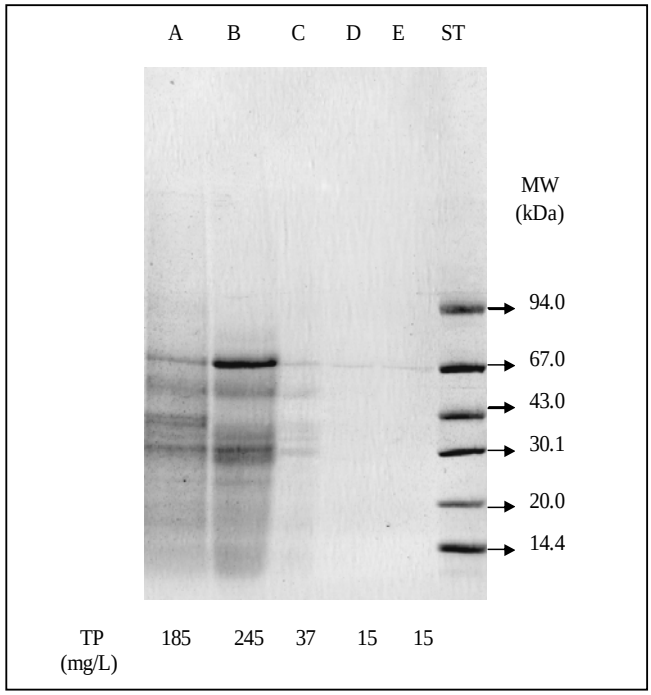

Fig. 3 Electrophoretic profiles of urinary proteins
in healthy full-term neonate
3, 7, 14, 21 and 28 days after birth (A,B,C,D,E)
TP: total urinary proteins in mg/L,
MW: molecular weight,
ST:Standard (Pharmacia LKB LMW calibration proteins)

obtain more information about the quantity and SDS-PAGE profiles in these subjects.

The results obtained in our study for total urinary concentration have shown the highest values for TP excretion detected on days 3 and 7 with range from 10-405 mg/L which decrease thereafter with range from 10-50 mg/L on days 21 and 28. There are only a few reports related to TP concentration in neonates with different methods used for TP determination. The results in our study was in close agreement with the findings from Miltenyi (22) who reported TP concentration between 95 - 455 mg/L in healthy full-term newborn infants in the first month of life (7-30 days old).

In our study the determination of proteine-to creatinine ratio was included, since the results of great number of studies of adults and children have shown that there is a strong correlation between the urine P/C ratio, obtained in random urine samples, and 24-hour urinary protein excretion corrected for body–surface area (24-26). Thus with determination of P/C ratio there is no need for 24-hour urine collection.

The highest value for P/C ratio in our study was detected in urine samples excreted on day 3 after birth (480 mg/g creatinine). In urine samples excreted on day 7 a similar values for P/C was determined as in samples excreted on day 3. In urinary samples excreted on day 14, 21 and 28 the values for P/C ratio were lower with highest detected values of 415 mg/g creatinine. It is obvious that the differences between values for P/C ratios between urinary samples excreted on day 3 and samples excreted on days 7,14, 21 and 28 was no so expressive like the differences noticed for otal protein concentration expressed in mg/L for the same urinary samples. In urine samples excreted on day 3 the values for total protein concentration ranged between 10-405 mg/L and decreased rapidly thereafter in urine samples excreted on day 14 (10-120 mg/L) with lowest values in samples obtained on days 21 and 28 (10-50 mg/L). These results confirmed the usefulness of urinary P/C ratio as a better measure for urinary protein concentration, since it is very obvious that dividing the total protein concentration by urinary creatinine concentration eliminates variations due to changing rates of urine output and provides a measure independent of urine concentration.

The results obtained in our study for P/C ratio was in close correlation with the results reported by other investigators. In a study of Houser (26) the upper value for P/C ratio found in 1 month old healthy infants was 500 mg/g creatinine, which value is very similar to the values in our investigated group of healthy neonates in first month of life.

The SDS-PAGE profiles obtained in our study from investigation of the physiological proteinuria in healthy full-term neonates reflected an incompletely developed renal function. The presence of numerous bands distributed over the broad range of molecular weights observed in urine samples from neonates could be explained with transitory immaturity of the glomerular filter and of the tubular protein reabsorbing system of the newborn kidney. Apparently, the SDS-PAGE profiles demonstrated that tubular protein handling normalizes later than the glomerular filtration of proteins.

The presence of protein fraction with molecular weight of about 160 kDa, which according to molecular weight belonged to imunoglobulin G, in some urinary samples of neonates excreted on days 3 and 7 after birth (Fig. 1), could be due to the fact that maternally derived antibodies are present at birth. Likewise, in the most of neonates prominent fraction with molecular weight of about 45 kDa was detected in urinary samples excreted on days 3 and 7 after birth, which according to molecular weight belonged to α_1- acid glycoprotein, probably due to the acute phase response at birth.

Electrophoretic SDS-PAGE profiles, obtained in our study, have shown that the intensity of albumin fraction (67 kDa) decreased during the neonatal period, which finding could be supported by the results from quantitative determination of albumin in urine samples of neonates, excreted during the first month of life, reported by Tsukahara et al (27). The results from their study have shown that urinary albumin decreased postnatally in healthy full-term neonates, while it remained almost constant in preterm neonates. In sick pre-

terms who were depressed at birth and have respiratory failure, urinary albumin was elevated during the first week, indicating the presence of glomerular damage in this period.

In almost all urinary samples excreted on days 3, 7 and 14 after birth, intensive fraction with molecular weight about 30 kDa was detected, which according to molecular weight belonged to α_1-microglobulin. This findings is in close correlation with the results from quantitative determination of α_1-microglobulin in urine samples of neonates, reported from Tsukahara et al. (28). The results from their study have shown high levels of α_1-microglobulin in urine samples of neonates excreted during the first two weeks of life, which declined thereafter. This finding is due to the fact that α_1-microglobulin was found by many authors to be early marker of renal tubular dysfunction, characterized by incomplete or inadequate reabsorption of low molecular weight proteins.

On SDS-PAGE profiles, obtained in our study, in a great number of urine samples from neonates, excreted on days 3 and 7, the presence of protein fraction with molecular weight of aproximately 12 kDa, with poor resolution, was found (Fig. 1 and Fig. 3). According to molecular weight this fraction belonged to β_2-microglobulin, which finding could be supported by results from quantitative determination of β_2-microglobulin in urine samples of neonates, during the first month of life, reported by Tsukahara et al (27). The results from their study have shown peak level on day 7 for β_2-microglobulin, both in term and preterm neonates, with trend towards higher levels of β_2-microglobulin with decreasing gestation, showing that proximal tubular protein reabsorption decreases with increasing degrees of prematurity. In sick preterms who were depressed at birth and had respiratory failure, this parameter was elevated during the first two weeks, indicating the presence of tubular damage in this period.

Electrophoretic SDS-PAGE profiles in 16 of neonates have shown the presence of visible, but with poor resolution protein fraction, with molecular weight of about 16 kDa. This fraction was found in all urine samples excreted on days 3,7 and 14 and in some urine samples excreted on days 21 and 28 as a palide fraction. According to molecular weight this fraction belongs to hemoglobin, which is the fraction considered to be physiological, due to the shortened life of erythrocytes (22).

Karlsson and Kristoffer (21) investigated the urinary protein pattern of pooled urine samples in the first year of life. They found a tubular type proteinuria in a pooled urine sample of eight 1- 4 week old newborns, while the protein pattern of the pooled urine of eight 4-6 month old infants was identical with the pattern of adults. Their study has shown that tubular proteinuria disappears gradually during the first years of life. Miltenye and his coworkers (22) fractionated the urinary samples from 20 full-term neonates (aged from 7 to 30 days) by sodium dodecyl sulphate gel discelectrophoresis (7.5 % polyacrylamide gels stained with 0.6 % Amido Black in 7 % acetic acid). The results from their study have shown that proteinuria was predominantly tubular, although in some urine samples high molecular weight protein fractions with molecular weight bigger than 100 000 kDa was detected, as well as middle molecular weight fractions (89 000 and 80 000 kDa). They reported also the presence of hemoglobinuria during the first weeks of life.

The results obtained by analysis of the molecular weights of urinary proteins in healthy newborns, separated by SDS-PAGE and stained with Coomassie Brilliant Blue G-250, reported by Thanner et al. (23) confirmed the findings of previous mentioned authors for presence of glomerulo-tubular imbalance in early life. According to the number of separated protein fractions and their relative concentration (expressed as percent of total protein concentration) they concluded that proteinuria in healthy full-term neonates is predominantly tubular and is due to ineffective proximal tubular reabsorption of low molecular weight microproteins.

Conclusion

The results in the present study, related to examination of SDS-PAGE profiles in healthy full-term neonates, confirmed the results obtained by other investigators that electrophoretic profiles are a source of great number of information about the physiological proteinuria frequently detected in urine of neonates by screening methods. Knowledge of the modifications that occur in urinary protein pattern due to age is therefore very important in order to distinguish such modifications from those caused by other factors. Investigation of SDS-PAGE profiles, coupled with determination of few parameters related to protein excretion, such as total urinary protein concentration and proteine-to-creatinine ratio might be of great usefulness for detection of early renal lesions in neonates, in clinical condititions in which renal function is affected.

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Резиме

Евалуација на уринарните протеини кај здрави доносени новородени со примена на SDS-PAGE

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Клучни зборови: Протеинурија, новородени, SDS-PAGE, електрофоретски профили

Извршено е испитување на видот на излачените уринарни протеини кај здрави доносени новородени со примена на натриум додецил сулфат полиакриламид гел електрофореза (SDS-PAGE), заедно со одредување на неколку параметри поврзани со екскрецијата на уринарните протеини.

Во испитувањето беа вклучени 20 здрави доносени новородени деца. Како материјал во испитувањето беа користени по пет примероци урина од секое новородено, излачени 3, 7, 14, 21 и 28 дена по раѓањето. Определувањето на вкупните протеини беше направено со турбидиметриски метод со сулфосалицилна киселина. Концентрацијата на креатининот во примероците урина беше определувана со методот по Jaffe. Сепарацијата на уринарните протеини беше направена со хоризонталната градиентна SDS-PAGE според Görg.

Највисоки вредности за вкупните уринарни протеини и за протеин/креатинин сооднос беа детектирани во примероците урина излачени третиот и седмиот ден по раѓањето. Беа утврдени три типа на електрофоретски профили. За првиот тип електрофоретски профили беше карактеристично присуството на протеини од мешано, гломеруларно и тубуларно потекло со молекуларна маса од 10 до 160 kDa. Типично за вториот тип електрофоретски профили беше присуството на две бледи фракции со молекуларна маса од 78 и 90 kDa, како и повеќе интензивни ниско молекуларни фракции (14-67 kDa). За третиот тип електрофоретски профили карактеристично беше присуството само на ниско молекуларни фракции (10-67 kDa) во сите пет испитувани примероци урина. Може да се заклучи дека наодите од испитувањето ја изразуваат минливата незрелост на гломеруларниот филтер и на системот за реапсорбција на тубуларните протеини кај бубрегот од новороденото. Очигледно е дека тубуларната функција се воспоставува подоцна во споредба со гломеруларната филтрација на протеините.

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Orally disintegrating tablet: formulation design and optimisation using Response Surface Methodology

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Abstract

The objective of this study was to develop diazepam orally disintegrating tablets and to optimize tablets characteristics using response surface methodology (RSM). Tablets were prepared by direct compression of mixture containing mannitol, copovidone, crosspovidone flavor and lubricant. A full factorial design for 2 factors at 3 levels each was applied to investigate the influence of 2 formulation variables on the mechanical strength/hardness, the percent of friability, disintegration time and dissolution of the poorly soluble active ingredient. The amounts of copovidone and crosspovidone were taken as independent variables. All data were analyzed by using statistical program.

The results of multiple linear regression analysis revealed that for obtaining a rapidly disintegrating dosage form, tablets should be prepared using an optimum concentration of crospovidone and copovidone. A contour plot is also presented to graphically represent the effect of the independent variables on the tablet hardness, disintegration time, percentage friability and dissolution. A checkpoint batch was also prepared to prove the validity of the evolved mathematical model.

3 level factorial design allowed us to obtain ODT with rapid disintegration and dissolution of the active ingredient with desirable properties of low tablet friability and appropriate mechanical strength (hardness) of the tablet.

Key words: orally disintegrating tablets, experimental design, response surface methodology.

Introduction

Recent developments in technology have presented viable dosage alternatives for patients who may have difficulty swallowing tablets. Traditional tablets and capsules may be inconvenient or impractical for some patients. Geriatric and pediatric patients experience difficulty in swallowing conventional tablets which leads to poor patience compliance. This problem is overcomed with use of drug delivery system known as orally disintegrating tablets. These are novel types of tablets which disintegrate/dissolve/disperse in saliva for 60 seconds or less (1, 2, 3).

They are also suitable for the mentally ill, the bedridden and patients who do not have easy access to water. The benefits in terms of patience compliance, rapid onset of action, possibility for increased bioavailability, and good stability make these tablets popular as a dosage form of choice in the current market (2). Orally disintegrating tablets are suitable formulation for all active substances which can be orally administered (3).

A pharmaceutical formulation is composed of several composition factors and process variables. These factors and variables not only affect the characteristic property of the dosage form but also make it difficult to formulate. Formulation experience with pharmaceutical preparation generally can guide a formulation expert to select those variables that most likely have an effect on those corresponding responses (4).

Traditional formulation designs were based on trial and error. It is time-consuming, unreliable, costly and often unsuccessful. The rapid development of statistical experimental designs, optimisation techniques and computer technologies provide an effective method for modelling

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complicated multivariate drug formulations. Pharmaceutical formulation thus has been brought into a new era (5).

When a target is to evaluate one or more tablet properties, the experimenter can make use of a set of tools and techniques known under experimental research methodology. In general, adopting such an experimental approach means defining the problem which one is going to cope with by determining the objectives, the possible constraints in the component proportion and the response variables under study (6).

Many statistical experimental designs have been recognized as useful techniques to optimize the process variables. Different types of designs as screening, mixture, response surface, have been used for preformulation evaluations (7, 8).

Response surface Methodology (RSM) is a widely practiced approach in the development and optimization of drug delivery devices. Based on the principal of design of experiments (DoE), the methodology encompasses the use of various types of experimental designs, generation of polynomial equations, and mapping of the response over the experimental domain to determine the optimum formulation. The technique requires minimum experimentation and time, thus providing to be far more effective and cost effective than the conventional methods of formulating dosage forms. It is a collection of statistical and mathematical techniques, useful for developing, improving and optimizing processes (9, 10). It also has an important application in the design, development and formulation of new products as well as in the improvement of existing product designs. The basic components of response surface methodology include experimental design, regression analysis and optimisation algorithms which are used to investigate the empirical relationship between one or more measured responses and a number of independent variables, with the ultimate goal of obtaining an optimal problem solution. Also, interaction between different factors, which can influence the target responses, may be detected (11, 12, 13).

The current study aims at developing and optimizing an orally disintegrating tablet of diazepam using RSM.

Experimental

Materials

Diazepam was supplied from Fabbrica Italiana Sintetici - Italy, Mannitol direct compressible grade from Merck KgA-Germany, Copovidone and Crosspovidone from BASF KgA, Germany and magnesium stearate from Mosselman, Belgium.

Methods

Experimental design

Response surface experimental design was used to optimize and evaluate main effects, interaction effects, and quadratic effects of the formulation ingredients on the percent of friability, hardness, disintegration time and drug release. A 3^2 randomized full factorial design is suitable for exploring response surfaces and constructing polynomial models (10).

In this design 2 factors were evaluated, each at 3 levels, and experimental trials were performed at all 9 possible combinations. The amount of the binding agent, copovidone (X1) and the amount of superdisintegrant, crosspovidone (X2) were selected as independent variables. The percent of friability, hardness, disintegration time and drug release were selected as dependent variables.

The composition of formulations of the factorial design is shown in Table 1.

Preparation of Diazepam orally disintegrating tablets

The raw materials were passed through a No.1.2 screen prior mixing. Diazepam, copovidone and crosspovidone were homogeneously blended with directly compressible mannitol as a diluent. The mixture than was blended with the fixed quantity of the lubricant magnesium stearate and compressed into flat faced tablets (6 mm diameter) using rotate tablet compression machine.

Table 1. Responce surface experimental design

Series	Diazepam (%, *w/w*)	Flavor (%, *w/w*)	Magnesium stearate (%, *w/w*)	Copovidone (%, *w/w*)	Crosspovidone (%, *w/w*)	Mannitol DC (%, *w/w*)
1	2.6	0.2	3.0	0.50	1.0	92.70
2	2.6	0.2	3.0	5.00	1.0	88.20
3	2.6	0.2	3.0	0.50	5.0	88.70
4	2.6	0.2	3.0	2.75	1.0	90.45
5	2.6	0.2	3.0	0.50	3.0	90.70
6	2.6	0.2	3.0	2.75	3.0	88.45
7	2.6	0.2	3.0	5.00	5.0	84.20
8	2.6	0.2	3.0	5.00	3.0	86.20
9	2.6	0.2	3.0	2.75	5.0	86.45

Evaluation of tablet properties

Hardness /Mechanical strength

The tablet hardness was measured by hardness tester (VanKelVK200 ,USA)

Percent Friability

The friability of tablets was determined using friability tester (VanKel, USP). The method of determination was according to USP28 method. The percent friability was obtained from 20-tablet sample.

Disintegration Time

The disintegration time was measured by using according USP 28 method for disintegration of tablets (VanKel 6-Basket System,USP)with purified water at 37 ^{o}C as disintegration medium. The mean and standard deviation were calculated from six determinations.

Percent Drug Release

Dissolution studies were performed employing USP 28 paddle method (VanKel Dissolution tester, VK-700, USA), 900 ml of 0.1mol/l HCl as dissolution medium at 50 rpm. The samples were analyzed using HPLC method (USP28).

Tablet assay

The tablets were assayed for drug content according USP 28 monograph for Diazepam tablets (Varian, HPLC system UV/vis variable wavelength detector, USA).

Statistical analysis

The data obtained from the evaluation of the tablet characteristics were analyzed using Statgraphics® plus, Windows software program (Version 5.1, USA).

Results and discussion

Tablet evaluation

All nine formulations of the experimental response surface design were prepared as different combinations of independent variables, copovidone (0.5-5 %) and crosspovidone (1-5 %) and the following parameters were analyzed: hardness, tensile strength, friability, disintegration and dissolution of the tablets.

The results of analysis of dependent variables- responses for each formulation are presented in Table 2.

The expressed results are mean values, for the hardness and friability determination of 20 tablets, disintegration of 12 and dissolution of 6 tablets.

During the preparation of the tablets, lower mechanical strength and increased friability for the series 1, 3, 5 under the applied pressure during tabletting were gained (RS design, quantity of the binding agent copovidone 0.5 %). Adequately, the friability of these formulations was not within the required limit, as described in USP28.

The hardness and friability were improved with increase of the concentration of the dry binder in the formulations. Complete release of diazepam from the prepared formulations was noticed after 20 and/or 30 min, showing differences in drug release rates due to different composition among series within the first 10 and 20 min. Disintegration of the tablets showing appropriate mechanical strength was fastest for series 9 (combination with 2.75 % of copovidone and 5 % of crosspovidone). However, the results given in Table 2 express that percent of drug release and the time of disintegration are not parameters that could be easily evaluated (followed) and are complex functions which mathematical expression and dependence (effect) of independent variable will be obtained from the experimental design.

Table 2. Results of the determination of the dependent variables for each series

Series	Y_1 [a] (kp)	Y_2 [b] (MPa)	Y_3 [c] (%)	Y_4 [d] (sec)	Y_5 (%) [e]		
					10 min	20 min	30 min
1	1	0.30	1.050	20	95.80	97.02	complete diss.
2	5.5	1.80	0.062	100	95.08	98.30	complete diss.
3	1	0.31	1.163	10	95.53	95.08	complete diss.
4	4	1.27	0.078	55	96.20	97.20	complete diss.
5	1	0.31	1.091	12	94.68	97.30	complete diss.
6	4	1.28	0.085	19	94.30	95.60	complete diss.
7	5.5	1.80	0.051	40	93.16	95.06	complete diss.
8	5.5	1.81	0.055	60	93.89	97.41	complete diss.
9	4	1.27	0.085	12	93.11	96.15	complete diss.

a Y1 - Hardness b Y2 - Tensile strength c Y3 - Friability d Y4 - Disintegratione Y5 - Dissolution of diazepam in 0.1 mol/l HCl

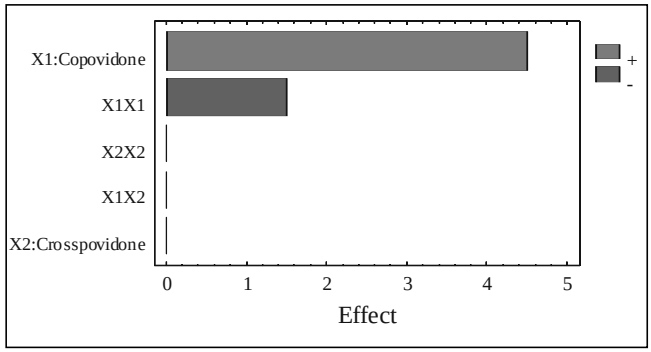

Fig. 1. Standard pareto chart showing the effects of independent variables X1 (copovidone) i X2 (crosspovidone) and their combined effects on the response Y1 (hardness of the tablets)

Factorial design

Hardness of tablets

The relationship between the hardness of the tablets and the independent variables is presented in Fig. 1.

The standardized pareto chart at Fig. 1. depicts the main effect of the independent variable on the tablet hardness. The length of each bar in the graph indicates the effect of these factors and the level of their responses. From Figure 1 it can be interfered that the factor X1 has a main effect on the response Y1. Factors X2, X1X2, X2X2 did not show significant effects on the Y1 response (the hardness of the tablets). With excluding of the factors which do not have significant effect on the response, the mathematical relationship in the form of polynomial equation for the measured response Y1 would be:

$$Y1=0.75+1*X1 \quad \text{(Eq. 1)}$$

Results presented in Fig. 1 and Eq. 1 show that the most significant effect on the hardness of the tablets has the binding agent copovidone, without the significant influence of the interaction between the factors *i.e.* between the binding and disintegrating agent.

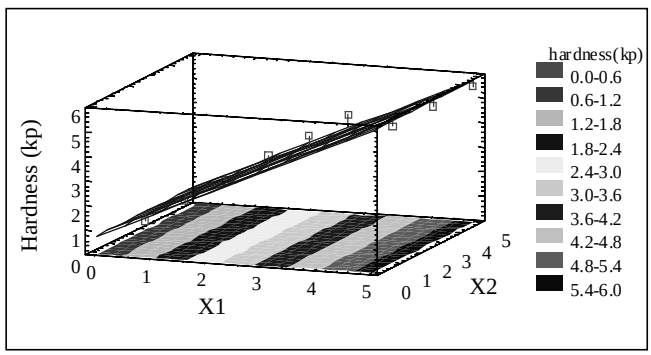

Fig. 2. Response surface plots showing the effect of independent variable X1 (copovidone) on response Y1 (hardness of the tablets)

Also, Fig. 2 illustrates that the quantity of the binding agent is a factor which shows significant and linear effect on the hardness of the tablets (when applied constant punch pressure).

Friability of the tablets

The mathematical relationship in the form of polynomial equation for the measured response, Y3 is listed below:

$$Y3=1.37834-0.750494*X1+0.0280278*X2+0.0979753*X1^2-0.00688889*X1X2 \quad \text{(Eq.2)}$$

The polynomial equation represents the quantitative effect of process variables (X1 and X2) and their interaction on the response Y3. The values of coefficients X1 and X2 are related to the effect of these variables on the response Y3. A positive value represents an effect that favor the optimisation, while a negative value indicates an antagonistic effect.

The main effect on the independent variables on the dependent variables was further investigated using a pareto chart (Fig. 3) and interaction plot (Fig. 4). Also, the relationship between the dependent and independent variables

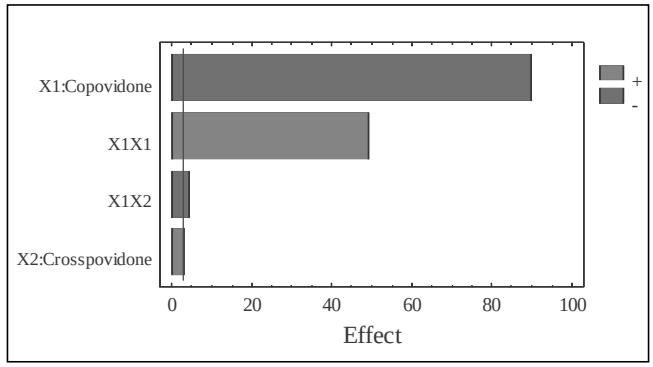

Fig. 3. Standard pareto chart showing the effects of independent variables X1 (copovidone) and X2 (crosspovidone) and their combined effects on the response Y3 (friability of the tablet)

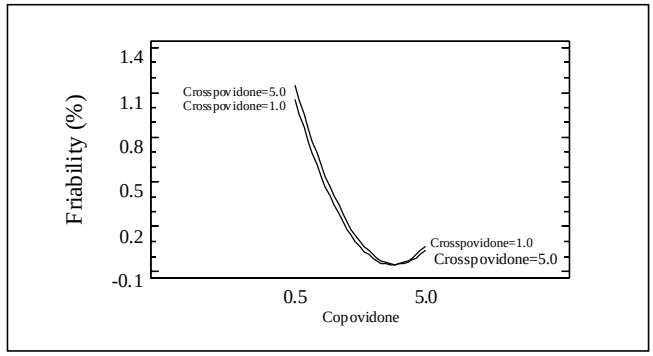

Fig. 4. Interaction plot showing the effects of interactions between the factors on the friability of the tablets

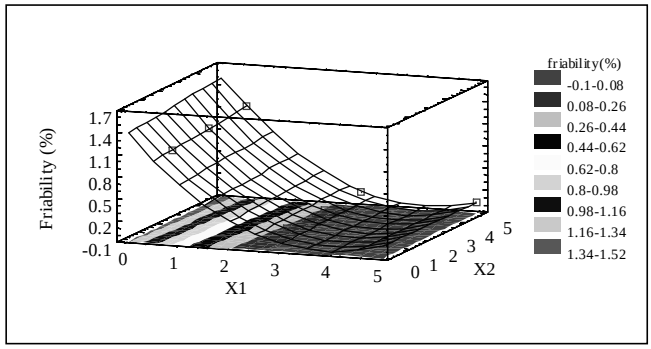

Fig. 5. Response surface plots showing the effect of independent variables, varying ratio of (X1) copovidone and (X2) crosspovidone on response Y3 (friability of the tablet)

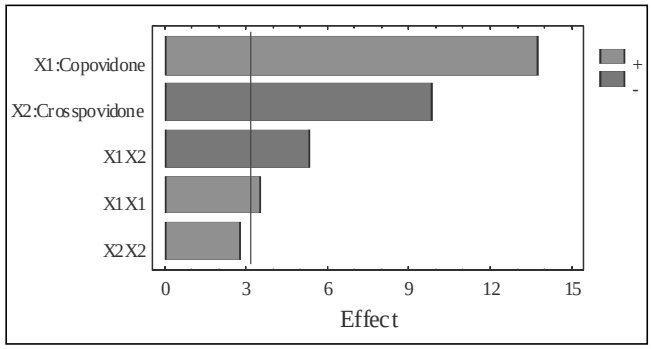

Fig. 6. Standard pareto chart showing the effects of independent variables X1 (copovidone) and X2 (crosspovidone) and their combined effects on the response Y4 (disintegration of the tablet)

was further elucidated using response surface plots (Fig. 5). Fig. 3 depicts the main effect of the independent variable on the friability of the formulations.

From Figs. 3 and 4 it can be interfered that the factors: X1, X1X1 (quadratic effect of copovidone), X1X2 (interaction effect of copovodone and crosspovidone) and X2 have a significant effect on the response Y3.

Fig. 5 further explains the effect of X1 and X2 ratios on the friability of the tablets. From response surface plots it is clear that the ratio of copovidone and crosspovidone has a major effect on determining friability of formulations. Figure 5 shows that at lower concentrations of copovidone the friability increases.

Disintegration of tablets

Fig. 6 represents the influence of independent variables on disintegration.

Mathematical relationship in a form of polynomial equation is listed below:

Y4=19.2428+7.36214*X1-1.77778*X2+2.30453*X1^2-2.77778*X1*X2 (Eq.3)

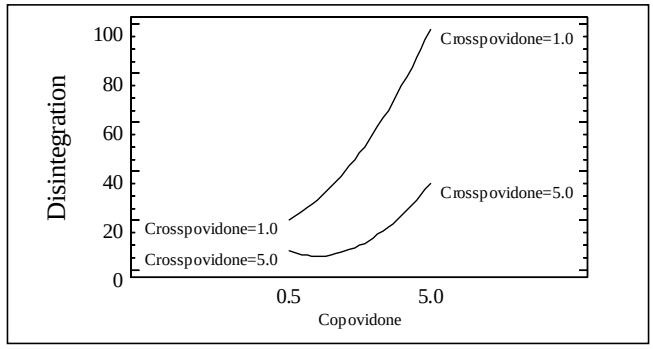

Fig. 7. Interaction plot showing the effects of interactions between the factors on the disintegration of the tablets

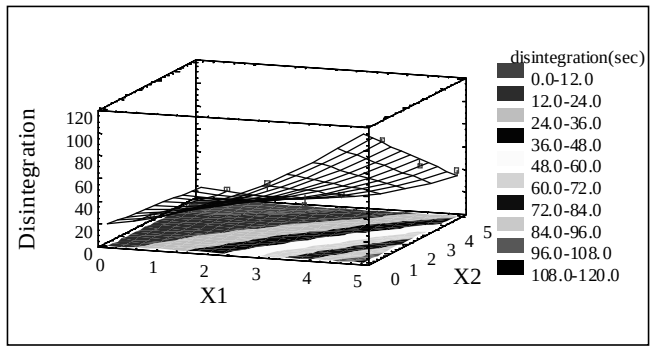

Fig. 8. Response surface plots showing the effect of independent variables, varying ratio of (X1) copovidone and (X2) crosspovidone on response Y4 (disintegration of the tablet

The main effect on the disintegration time depend on copovidone and crosspovidone quantity in the formulation, interaction between the binding and disintegrating agent and quadratic effect of copovidone. Decreasing the percentage of copovidone and increasing the percentage of crosspovidone decreases the time of disintegration of the tablets (influence of the copovidone/crosspovidone interaction on the disintegration time is shown on Fig. 7).

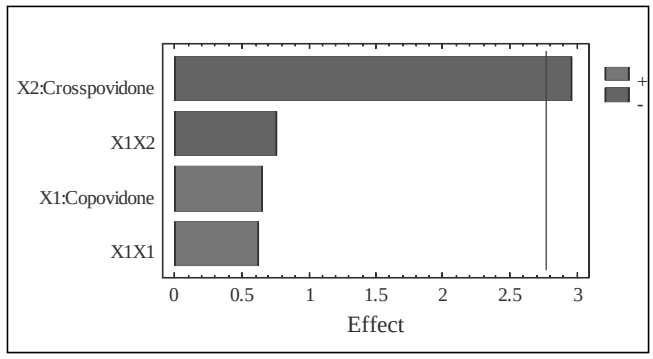

Fig. 9. Standard pareto chart showing the effects of independent variables X1 (copovidone) and X2 (crosspovidone) and their combined effects on the response Y5 (dissolution of diazepam in 0.1mol/l HCl, for 30 minutes)

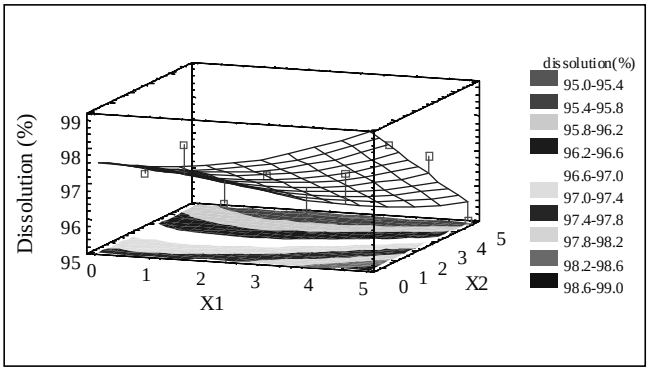

Fig. 10. Response surface plots showing the effect of independent variables, varying ratio of (X1) copovidone and (X2) crosspovidone on response Y5 (dissolution of diazepam in 0.1 mol/l HCl)

Adequately, at level of 1 % of incorporated disintegrating agent, increasing quantity of copovidone will significantly increase the value of the disintegration time for the prepared formulations (Fig. 8 shows response surface plots showing the effect copovidone and crosspovidone on disintegration).

Drug dissolution

Dissolution of the active ingredient in orally disintegrating tablets starts in the mouth after the fast disintegration where the pH is approx. 6.8 (6.4-7.2). After the swallowing of the granules or the primary particles the drug dissolution continues at pH approximately 1.5 and the absorption of the released drug depends on the basic dissolution of the active ingredient and its permeability through the membranes. Diazepam is classified in the second group of biopharmaceutical classification system i.e. is practically insoluble substance at pH 6.8 or at basic pH and showing good permeability through the biological membranes. Except better patient compliance, fast dispersible/disintegration tablet means avoiding the factors of the disintegration process on the efficacy of the tablet formulation. As diazepam dissolution rate decreases at basic pH, its immediate availability in primary particles for dissolution after reaching the gaster and pH 1.5 might contribute to its better availability for absorption.

The Response Surface design shows that drug release in acidic medium is influenced only by the quantity of disintegrating agent, which is represented in Fig. 9 and Fig. 10 where factor X2 shows the main significant effect.

Conclusion

Optimization of Diazepam ODT using response surface full factorial design was performed.

The quantity and the ratio of independent variables showed significant effects on the tablet characteristics.

The most significant effect on the hardness of the tablets shows the binding agent copovidone. Increasing the quantity of copovidone increases the hardness of the tablets.

The most significant effect on the friability of the tablets has copovidone, although effect on the friability due to the interaction between copovidone and crosspovidone is also present. With increasing the content of copovidone the percent of friability of the tablets decreases.

The quantity of the copovidone and crosspovidone presents the main effect on the tablet disintegration. Interaction among copovidone and crosspovidone shows significant effect on the disintegration process, also. Increasing the quantity of crosspovidone and decreasing the quantity of copovidone decreases the time of disintegration of the tablets. Faster disintegration of diazepam tablet means that complete drug release will be achieved in medium where the active substance has a good solubility i.e. the influence of the disintegration time of the pharmaceutical dosage form on the dissolution time and of the active component is completely avoided and the dissolution rate and time will depend only on the characteristics of the active ingredient, which if needed might be modified as well. Based on the quantitative effect of the polynomial equations generated by RSM, the optimum formulation from RS design could be formulation with adequate hardness, friability and disintegration time less than 20 sec. Such formulation/s contains copovidone 2.75 % and crosspovidone in a quantity of 3-5 %.

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Резиме

Перорална дисперзибилна таблета: Формулациски дизајн и оптимизација со примена на Response Surface методологија

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Клучни зборови: перорална дисперзибилна таблета, експериментален дизајн, response surface методологија.

Цел на овој труд беше развој на диазепам перорална дисперзибилна таблета и оптимизација на карактеристиките на таблетата со примена на response surface методологијата. Таблетите беа подготвени со директна компресија на мешавина од манитол, коповидон, кросповидон, арома и средство за лубрикација.

Беше применет full factorial дизајн на два фактори на формулацијата, секој поставен на 3 нивоа, со цел да се испита ефектот на двата променливи фактори на формулацијата врз механичката јакост/цврстината, процентот на фријабилност, времето на дезинтеграција на таблетите и дисолуцијата на тешко растворливата активна компонента. Како независни променливи беа земени количините на коповидон и кросповидон. Добиените резултати беа анализирани со примена на соодветна статистичка програма.

Резултатите од мултипната регресија покажаа дека за да се добие дозирана форма со многу брза дезинтеграција, таблетите треба да содржат оптимална концентрација на коповидон и кросповидон.

Ефектот на независните променливи на цврстината на таблетата, времето на дезинтеграција, процентот на фријабилност и дисолуцијата графички е претставен со соодветни контурни нацрти. За да се прикаже валидноста на применетиот математички модел беше подготвена и контролна серија.

Факторијалниот дизајн на 3 нивоа овозможи да се добие перорална дисперзибилна таблета со многу брза дезинтеграција и дисолуција на активната компонента, со задоволителна фријабилност и соодветна механичка јакост (цврстина) на таблетите.

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Original scientific paper

HPLC determination of hydrochlorothiazide in urine after solid-phase extraction

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Abstract

A simple, rapid and precise HPLC method has been developed for the assay of hydrochlorothiazide in urine. The clean-up of the urine samples was carried out by solid-phase extraction using HLB cartridges. Extraction recovery was 94.00-100.28 %. HPLC separation was performed with isocratic elution on Hypersil BDS C18 column (100 x 4.0 mm I.D., 3 µm particle size) protected with appropriate guard column. The mobile phase was 18 % acetonitrile and 0.025 mol/L solution of KH_2PO_4, pH 4 at flow rate of 0.3 mL/min. Detection of the substances was performed at 220 nm. The calibration curves were linear in the range of 2-50 µg/mL. The developed method is validated by checking its accuracy, precision and stability. The detection limit is 2 µg/mL hydrochlorothiazide. The method is proved to be convenient for routine analysis of hydrochlorothiazide in urine.

Keywords: Hydrochlorothiazide, urine, solid-phase extraction, HPLC

Introduction

Hydrochlorothiazide is a thiazide diuretic drug (Fig. 1), which is used in treatment of hypertension, either alone or together with other agents, it is effective in treatment of congestive heart failure and hepatic disorders. In medical practice, it is mostly used in a conventional tablet form in dose of 25 to 250 mg (1).

Several investigations have been performed for determination of hydrochlorothiazide in urine by high-performance liquid chromatography (HPLC) with UV detection and electrochemical detection. Zendelovska and Stafilov (2) published a review of HPLC methods for determination of diuretics in biological fluids including procedures for sample preparation. Liquid-liquid extraction with different organic solvents is mostly used for determination of hydrochlorothiazide in urine. But, in this way, separation of hydrochlorothiazide from interferential components is not achieved and recovery values are poor. Cooper and coworkers (3) suggested a liquid-liquid extraction of hydrochlorothiazide from urine and serum by ethyl acetate and back extraction with sodium hydroxide, but recovery values were 91,5 ± 2,5 %. Shiu and coworkers (4) proposed a liquid-liquid extraction of hydrochlorothiazide from urine with ethyl acetate, but again, the recovery values are ranged from 62.2 to 80.7 %. Alton and coworkers (5) have also used ethyl acetate for extraction of hydrochlorothiazide from urine.

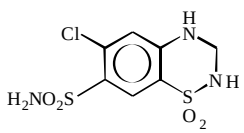

Fig. 1. Structure of hydrochlorothiazide

On the other hand, Domingo and coworkers (6) used micellar liquid chromatography for determination of hydrochlorothiazide in urine by direct injection of sample in the chromatographic system without any sample pretreatment. Zendelovska and coworkers (7) developed a solid-phase extraction method for determination of hydrochlorothiazide in human plasma. Papadoyannis and coworkers (8, 9) have performed a chromatographic deter-

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mination of cephalosporin antibiotics in biological fluids and pharmaceuticals using hydrochlorothiazide as an internal standard. The confirmation technique most widely used is GC/MS, which requires methylation of the aminosulfonyl group followed by GC/MS measurement (10).

The purpose of this study was to develop and validate a liquid chromatographic method with UV detection suitable for the determination of hydrochlorothiazide in urine. A new solid-phase extraction method was developed using HLB cartridges. This is a convenient method for separation and concentration of the analyzed drug and elimination of urine interferences, because of its efficiency and rapid sample preparation. The method was validated by evaluating the recovery, selectivity, linearity, precision and accuracy. Finally, the method was used for determination of hydrochlorothiazide in urine samples from patients.

Experimental

2.1. Materials and reagents

Hydrochlorothiazide was kindly provided from Changzhou Pharmaceutical Factory (China). Acetonitrile, methanol, phosphoric acid, and potassium dihydrogen phosphate were from Merck (Darmstadt, Germany). All solvents used were of HPLC grade. HPLC column was purchased from Hewlett Packard and cartridges for solid-phase extraction were purchased from Waters.

2.2. Preparation of standards

Stock solution of hydrochlorothiazide was prepared at concentration of 2 mg/mL by dissolving appropriate amounts of hydrochlorothiazide in methanol. The working solutions were prepared by diluting appropriate portions of this solution with redistilled water and stored at 4°C.

2.3. Instrumentation and HPLC analysis

Analysis of hydrochlorothiazide was carried out with a Varian HPLC system equipped with a ternary pump Model 9012 and UV Diode Array Detector Model 9065. Satisfactory results (peak shape and run time) were obtained with Hypersil BDS C18 column (100 x 4.0 mm I.D., 3 µm particle size) protected with appropriate guard column. A mobile phase consisting of solution of KH_2PO_4 with concentration 0.025 mol/L (pH 4) and 18 % acetonitrile was found to give the best results. Isocratic elution was performed with flow rate of 0.3 mL/min at ambient temperature. Elution was monitored in the whole UV region and the best detection was achieved on wavelength of 220 nm.

Solid-phase extraction was performed with the device (SPE) Visiprep™ from Supelco.

2.4. Sample preparation

Urine samples were collected from healthy volunteers and stored at -20°C. After thawing, samples were spiked daily with working solutions of hydrochlorothiazide. Several cartridges with different stationary phases for SPE were tested: LiChrolut RP-18 (Merck), LC-18 (Supelco) and Oasis HLB (Waters). Best results for isolation of hydrochlorothiazide were obtained when SPE was performed with Oasis HLB cartridges.

Before extraction, 1 mL of urine sample was buffered with 1 mL of 0.1 mol/L KH_2PO_4 solution, pH 9. The cartridges were conditioned with 1 mL methanol and 1 mL redistilled water. The buffered urine was introduced into the cartridge. 1 mL of redistilled water was used to rinse the cartridge and remove the interfering components. Elution was performed with 1 mL methanol. The eluate was evaporated to dryness under air for about 15 min at 40-45°C. Reconstitution was performed with 200 µL mobile phase and 20 µL sample was injected into the HPLC system.

2.5. Calibration standards

Linear regression analysis was performed. Typical calibration curves were constructed with five blank urine samples spiked with appropriate amounts of the standard solution of hydrochlorothiazide. The calibration range was 2-50 µg/mL of hydrochlorothiazide in urine. Three quality control standards containing 7, 15 and 30 µg/mL hydrochlorothiazide in urine were used for precision and accuracy measurements.

Results and discussion

3.1. Method development

In order to develop a convenient, simple and rapid HPLC method for quantitative determination of hydrochlorothiazide in urine, several HPLC method variables with respect to their effect on the place and shape on the peak of hydrochlorothiazide, were investigated. The optimization of the HPLC conditions was performed for hydrochlorothiazide and cefotaxime, which was tested as an internal standard. Symmetrical shape of the peaks and relatively short time for analysis were obtained when separation was performed on Hypersil BDS C18 column (100 x 4.0 mm I.D., 3 µm particle size) protected with appropriate guard column with flow rate of 0.3 mL/min flow rate. The elution was monitored in the whole UV region and the wavelength of 220 nm exhibited the best detection of hydrochlorothiazide due to an absorption maximum at this wavelength.

Series of mobile phases with variable content of the organic modifier acetonitrile in range from 15 to 30 % were prepared in order to investigate its effect on hydrochloro-

thiazide and cefotaxime retention. Satisfactory results (peak shape, short retention time) were obtained when the volume fraction of acetonitrile in the mobile phase was 18 %.

Additionally, the effect of the buffer concentration on analyte retention was investigated in the concentration range from 0.0125 to 0.0500 mol/L. Best results were achieved with solution of KH_2PO_4 with concentration of 0.025 mol/L. The subsequent increasing of the buffer concentration caused increase in analysis time. Therefore, all following investigations were performed with a mobile phase, containing KH_2PO_4 with concentration of 0.025 mol/L and 18 % acetonitrile.

The effect of the pH value on the hydrochlorothiazide and cefotaxime retention was also checked by elution with mobile phases with pH in the range from 2.2 to 4.5. A mobile phase with pH 4 provided a short analysis time and good peak shape.

Finally, the effect of temperature on retention of the analyzed component was checked in the range from 20 to 50°C. The obtained results showed that temperature does not affect the peak shape and analysis time significantly, so ambient temperature was chosen for all investigations.

From these data it was established that the best results (satisfactory retention, acceptable runtime less than 6.5 min.) were obtained using mobile phase consisting of 18 % acetonitrile in solution of KH_2PO_4 with concentration of 0.025 mol/L and pH 4. The retention time of hydrochlorothiazide is 5.8 min and 4.0 min for cefotaxime. Typical chromatogram obtained from standard solution of hydrochlorothiazide and cefotaxime produced by the developed HPLC method is shown in Fig. 2.

Calibration diagram was constructed for establishing the dependence of the peak area of hydrochlorothiazide and concentration. The obtained linear dependence of peak area of hydrochlorothiazide and mass concentration of hydrochlorothiazide in pure solutions, with correlation coefficient (0.9999) is the following:

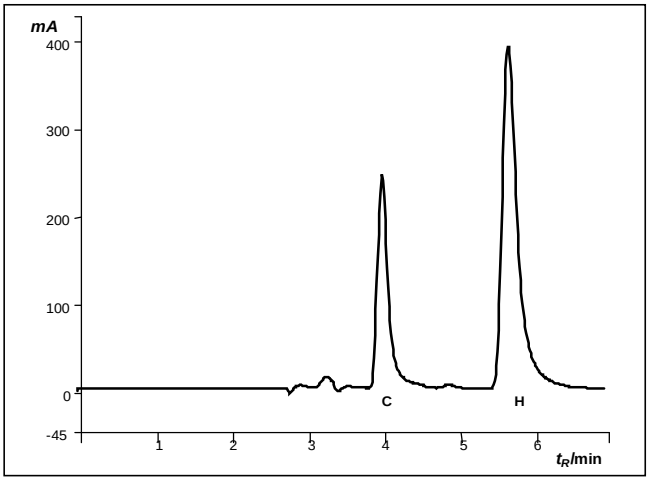

Fig. 2. Chromatogram of standard solution of hydrochlorothiazide (8 μg/mL) and cefotaxime (8 μg/mL)

$$Area(H) = 194029 \cdot \gamma(H)\ (\mu g/mL) + 297658$$

As previously said, several cartridges for solid-phase extraction (LiChrolut RP-18 (Merck), LC-18 (Supelco) и Oasis HLB (Waters)) were tested in order to obtain satisfactory values for recovery of hydrochlorothiazide from urine samples. Results from this investigation show satisfactory values for recovery of hydrochlorothiazide when urine samples were extracted on Oasis HLB (Waters) cartridges. The values obtained for recovery of hydrochlorothiazide ranged from 94.00 to 100.28 %. The chromatographic peak is sharp, with relatively symmetric shape with small tailing, well separated form the matrix components present in the urine (Fig. 3). In this phase, cefotaxime was excluded as a possible internal standard because of its poor separation from the endogenous compounds from urine and the method was then validated using HLB cartriges for sample preparation and the optimized HPLC conditions for measurement using calibration with an external standard of hydrochlorothiazide.

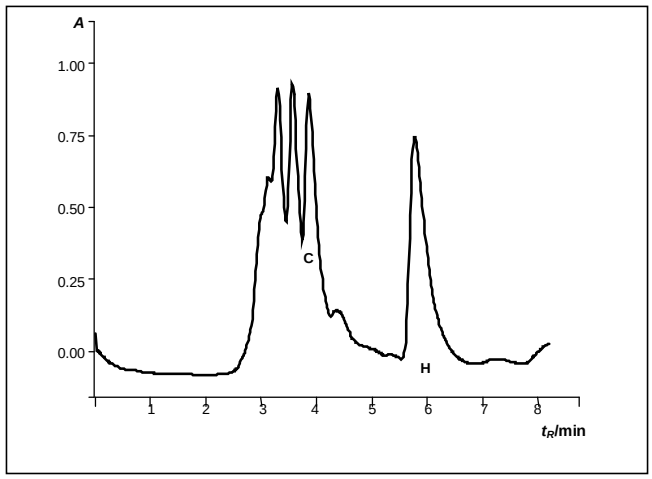

Fig. 3. Chromatogram of urine sample spiked with hydrochlorothiazide (25 μg/mL) obtained after SPE on HLB columns

Separation of interferential components from matrix was not achieved with application of RP-18 cartridges for solid-phase extraction of hydrochlorothiazide and a loss of analyzed component during the washing step was noticed. LC-18 cartridges were not convenient for separation of the analyzed component from matrix components present in urine. Endogenous components from urine were not eliminated and the chromatographic peak of hydrochlorothiazide was overlapped with chromatographic peaks of the interferential endogenous components.

Table 1. Intra- day and inter- day accuracy and precision data for hydrochlorothiazide in urine determined with solid-phase extraction

	Intraday precision and accuracy			Interday precision and accuracy		
	<γ>/ μg/mL n=3	*RSD* (%)	e_r (%)	<γ>/ μg/mL n=6	*RSD* (%)	e_r (%)
7 μg/mL	6.84	1.03	-2.24	6.86	5.16	-1.90
15 μg/mL	15.03	1.01	0.22	15.28	4.42	1.85
30 μg/mL	29.96	2.77	-0.13	30.05	3.61	0.16

3.2. Method validation

3.2.1. Linearity

Linearity was tested in 3 days at five concentration points ranging from 2 to 50 ?g/mL of hydrochlorothiazide. The following values for the slope, intercept, and correlation coefficient were obtained:

Slope = 184199.4 Intercept =- 46177.9 R^2 = 0.99977

3.2.2. Accuracy and precision

Intra-and inter-day precision and accuracy were determined by measuring urine quality control samples at low (7 μg/mL), middle (15 μg/mL) and high (30 μg/mL) concentration levels of hydrochlorothiazide. An indication of accuracy was based on calculation of the relative error of the mean observed concentration compared with the nominal concentration. Precision was expressed as relative standard deviation (RSD). Obtained results are presented in Table 1.

Relative errors ranged from 0.26 to 9.28 %, and relative standard deviation from 1.01 to 5.16 %. The obtained results indicated good precision and accuracy of the developed method.

3.2.4. Limit of quantification (LOQ)

The limit of quantification is defined as the lowest concentration of hydrochlorothiazide which can be measured with an error less than 20 %. The LOQ was determinated using the lowest calibration standard in five different analytical days. The RSD value of 7.57 % and a relative error less than 10 % confirmed that the hydrochlorothiazide concentration of 2 μg/mL in urine can be accepted as LOQ.

3.2.5. Stability of hydrochlorothiazide in urine samples

The stability of hydrochlorothiazide in urine samples was investigated with spiked samples at two different concentration levels prepared in duplicate. Spiked samples were analyzed after different storage conditions: immediately, after staying for 2 and 24 hours at room temperature, after one and two freeze/thaw cycles and after 1 month stored at -20°C. The obtained results form these investigations are shown in Table 2.

Table 2. Stability of hydrochlorothiazide in urine

Analyzed samples	7 μg/mL	e_r (%)	30 μg/mL	e_r (%)
Immediately	6.98	- 0.28	29.48	- 1.73
After 2h	6.72	- 3.72	30.95	4.98
After 24h	6.85	- 1.86	29.42	- 0.20
Cycle 1	7.02	0.57	31.12	5.56
Cycle 2	7.23	3.58	27.83	- 5.59
After 1 month	7.13	2.14	30.19	2.41

The obtained results show that relative errors are between 0.28 to 5.59 %, which means that, hydrochlorothiazide added in urine samples is stable under different storage conditions.

3.2.6. Ruggedness of the method

Ruggedness of the method was checked by employing the proposed HPLC method with slightly changed pH value of the mobile phase and by using slightly changed detection wavelength. The relative error was calculated by comparing the results obtained with the optimized conditions and the ones obtained with the slightly changed parameters (pH value of the mobile phase from 3.7 to 4.3, detection wavelength 215 and 224 nm). For this test, a standard solution with medium concentration of hydrochlorothiazide (7 μg/mL) was directly injected into the HPLC system. Results are present in Table 3.

The relative errors between 0.08 and 4.77 % indicating ruggedness of the developed HPLC method when using

Table 3. Ruggedness of the method for hydrochlorothiazide at slightly varied pH values and detection wavelengths

Sample	7 μg/mL	
	<Area>	e_r(%)
220 nm	2058948	
215 nm	1999378	- 2.89
224 nm	2137594	3.82
pH 2.5	2058948	
pH 2.2	2075528.3	0.81
pH 2.8	1990904	- 3.30

slightly variable pH values of the mobile phase. The results presented in Table 3, show that little changes in the detection wavelength produce relative errors in the range from 1.09 to 4.87 % which confirms the ruggedness of the SPE method developed for solid-phase extraction of hydrochlorothiazide.

The developed HPLC method after solid-phase extraction of hydrochlorothiazide from urine was used for analysis of patient urine samples after oral administration of the analyzed drug. Typical chromatograms obtained from urine samples prepared according to the developed procedure for sample preparation are shown in Fig. 4.

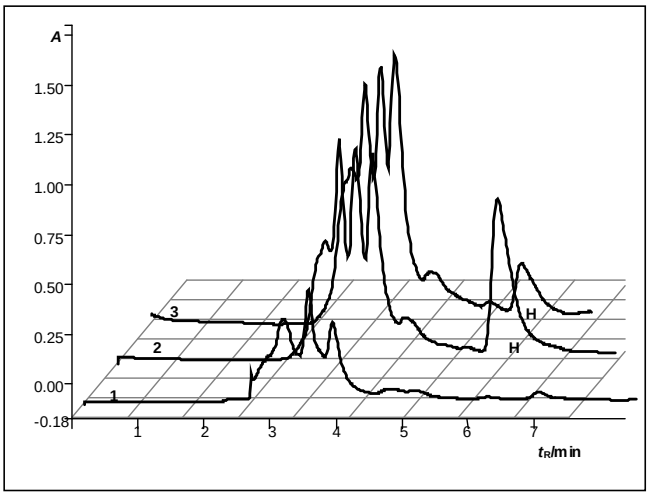

Fig. 4 Chromatograms obtained after SPE of:
1 - blank sample, 2 - spiked urine containing 25 µg/mL hydrochlorothiazide and 3 - patient urine sample (H - hydrochlorothiazide)

Conclusion

The HPLC method including solid-phase extraction for sample preparation is simple, rapid and convenient for determination of hydrochlorothiazide in urine samples. HPLC conditions are optimized as well as the sample preparation procedure. Different SPE cartridges for separation and concentration of hydrochlorothiazide were tested and best results are obtained with HLB cartridges. SPE conditions with HLB cartriges are optimized for separation of hydrochlorothiazide and efficient clean up of the complex biological matrix and high recovery of the investigated drug is achieved. The validation data demonstrate good precision and accuracy, which proves the reliability of the proposed method. Finally, this method is suitable for identification and quantification of hydrochlorothiazide in urine and it can be used in pharmacological studies.

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Резиме

HPCL определување на хидрохлортиазид во урина после цврстофазна естракција

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Клучни зборови: хидрохлортиазид, урина, цврстофазна, екстракција, HPCL.

Развиен е едноставен, брз и прецизен HPCL метод за определување на содржина на хидрохлортиазид во урина. Подготовката на примероците од урина и нивно прочистување е направено со HLB колони од цврстофазна екстракција. Приносот по пречистувањето е 94,00 – 100,28%. Раздвојувањето на компонентите е направено со искратско елуирање на Hypersil BDS C18 колона (100 x 4,0 mm, I.D., 3 μm големина на честички) со соодветна предколона. Мобилна фаза во состав: 18% ацетонитрил и 0,02 mol/L раствор на KH_2PO_4, pH 4 и проток на 0,3 mL/min. Детекцијата на супстанците е извршена на 220 nm. Калибрационата крива покажува линеарност во опсегот од 2-50 μg/mL. Методот е валидиран со проверка на точност, прецизност и соодветност на системот. Лимитот на детекција е 2μg/mL хидрохлортиазид. Овој метод веќе ја има докажано својата едноставност и употребливост во рутинска анализа на хидрохлортиазид.

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Original scientific paper

Antioxidant potential of *Helichrysum plicatum* DC. (Asteraceae)

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Abstract

The present study describes the ability of different extracts of *H. plicatum* obtained from flowers, stems and leaves, to act as natural antioxidants in different *in vitro* experimental models in which free radical reactions are involved: inhibition of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, inhibition of hydroxyl radicals and protection of β-carotene-linoleic acid model system.

Investigate extracts showed radical scavenging activity with IC_{50} from 6 to 11 mg/ml. The extracts are capable to reacting with $OH^{\bullet}$ radical with inhibition of its production ranged between 33-58%. The high preventive activity against the bleaching of beta-carotene (15-49% of initial value after 120 minutes) was also observed. The antioxidative activity of the extracts in the experimental systems was compared with that of reference substances: luteolin, quercetin, BHA, BHT and sylimarin (the main agent of the well-known milk thistle – *Silybum marianum* L.).

Results of this study suggest that *Helichrysum plicatum* represent a natural source with antioxidant potential.

Key words: *Helichrysum plicatum*, flavonoids, DPPH, hydroxyl radicals, lipid peroxidation.

Introduction

Helichrysum species (Asteraceae) have been well known plants in folk medicine for hundreds of years. One of them, *Helichrysum plicatum* DC. is widely distributed throughout the whole territory of Balkan Peninsula. Water extract (infuse) of this plant have been used for treatment of gastric and hepatic disorders, usually in combination with other plants with similar effects (1). Phytochemical screening (HPLC) of the *Helichrysum plicatum* from Macedonia proved the presence of apigenin and naringenin as free aglycones and glycosides of apigenin, naringenin, quercetin and kaempferol in the flowers as well as quercetin and luteolin glycosides and free luteolin in stems and leaves (2). Its antioxidant and scavenging properties have not been studied previously. Among phytochemicals, flavonoids deserve a special mention due to their free radical scavenging activities and *in vivo* biological activities that are being investigated by many researchers (3,4,5,6,7). The goal of research on antioxidative characteristics of plant extracts is to discover a potential replacement for synthetic antioxidants (BHT, BHA), which cause unwanted processes after prolonged used.

The evaluation of plant extracts antioxidant capacity is not easy task, as many methods can be used to determine this activity, and substrates, conditions, analytical methods, and concentrations can affect the estimated activity (8). This paper reports a study in which antioxidative activity of methanolic, ethyl acetate, and after hydrolysis extracts are tested. This effect was followed by three complementary *in vitro* methods: evaluation the free radical scavenging capacity (DPPH method) (9), inhibition of hydroxyl radicals production (10) and protection of β-carotene-linoleic acid system (11).

Materials and Methods

Plant material

The aerial parts of *Helichrysum plicatum* DC. were collected on the Golac Mountain, Eastern Macedonia, in the flowering period of the plant, during the summer of

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2001. Voucher specimens were deposited at the Herbarium of the Institute of Pharmacognosy, Faculty of Pharmacy, Skopje, Macedonia.

Reagents

The reagents used were of highest purity (>99.95 purity) and were purchased from Sigma Chemical Co (Germany). Authentic samples of luteolin, quercetin, sylimarin, BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) were the products of Extrasynthese (France).

Preparation of plant extracts

Dried powdered plant material (flowers, stems and leaves separately) was cut into small pieces and extracted in the ratio 1:10 (w/v) by three procedures: a. with methanol (M-extract); b. with ethanol-water (7:3), then evaporated until water remains and extracted with ethyl acetate (EA-extract); c. hydrolysis with HCl, aglycones extracted with ethyl acetate (H-extract). All extracts were dried over anhydrous sodium sulfate, filtered and concentrated under vacuum up to concentration of 1 g per 1 mL of extract. M-extract was further diluted with methanol; EA and H extracts with ethyl acetate, in order to obtained 0.01 g mL^{-1} solutions used in the experiments.

Free radical scavenging activity in 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) assay

The antioxidant activity using the DPPH assay was assessed by the method of Brand-Williams et al. (9). A test sample solution (200 μL) was added to 4 mL of 100 mmol L^{-1} ethanolic DPPH. After vortexing, the mixture was incubated for 10 minutes at room temperature and the absorbance at 517 nm was measured. The difference in absorbance between a test sample and a control (ethanol) was considered as activity. The activity was shown as IC_{50} value (50% of inhibitory concentration in mg mL^{-1}). Luteolin, quercetin, BHA, BHT and sylimarin (100 μg mL^{-1} in ethanol) were used as reference substances. All values are shown as the mean of three measurements.

Evaluation of the hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was carried out by measuring the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. Attack of the hydroxyl radical on deoxyribose led to TBARS (thiobarbituric acid-reactive substances) formation (10). The extracts were added to the reaction mixture containing 2.8 mmol L^{-1} deoxyribose, 100 μmol L^{-1} $FeCl_3$, 104 μmol L^{-1} EDTA, 100 μmol L^{-1} ascorbic acid, 1 mmol L^{-1} H_2O_2 and 230 mmol L^{-1} phosphate buffer (pH 7.4), making a final volume of 1.0 mL. One milliliter of thiobarbituric acid TBA (1%) and 1.0 mL trichloroacetic acid (TCA 2.8%) were added to test the tube and incubated at 100ºC for 20 min. After cooling, absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Reactions were carried out in triplicate. In the series of control experiments, reference substances: luteolin, quercetin, BHA, BHT and sylimarin (100 μg mL^{-1} in phosphate buffer-pH 7.4) were used instead of the extract solution. The reaction mixture was incubated at 37ºC for 1 h.

Evaluation of antioxidant activity

The antioxidant activity of the extracts was evaluated using a β-carotene/linoleate model system (11). A solution of β-carotene was prepared by dissolving 2.0 mg of β-carotene in 10 mL of chloroform. One milliliter of this solution was then pipette into a round-bottom flask. After chloroform was rotary evaporated at 40 ºC under vacuum, 20 mg of purified linoleic acid, 200 mg of Tween 40 emulsifier and 50 mL of distilled water were added to the flask with vigorous shaking. Aliquots (5 mL) of this emulsion were transferred into a series of tubes containing 2 mg of each extract or 2 mg of BHA (butylated hydroxyanisole) for comparison. An aliquot (5 mL) of emulsion without any further additions was used as control. As soon as the emulsion was added to each tube, the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded at 10-min intervals by keeping the sample in a water bath at 50 ºC until the visual color of β-carotene in the control sample had disappeared (about 120 min).

Results and discussion

Interest in the search for new natural antioxidants has grown dramatically over the past years because reactive oxygen species production and oxidative stress have been shown to be linked to ageing related illnesses (12). Also the restrictions to use of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), due to their toxicity (13) has been an important incentive for such research. Numerous plants have been examined for antioxidant activity. According to this, antioxidant properties of *Origanum* spp. (aerial parts) have been studied relatively well over the past years (14,15,16). Also, *Urtica* sp., has been shown to have antioxidant activity (17). On the other hand, *Helichrysum* species have not been investigated systematically for their potential health-benefiting properties.

Free radical scavenging activity

This method is based on the reduction of DPPH, a stable free radical. Because of the odd electron of DPPH, it gives a strong absorption maximum at 517 nm by visible

spectroscopy (purple color). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, that is, a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting discoloration is stehiometric with respect to the number of electrons captures (18). This reaction has been widely used to test the ability of compounds to act as free-radical scavengers of hydrogen donors and to evaluate the antioxidant activity of food and plant extracts (19-21).

Free radical scavenging activity (FRSA) of *Helichrysum* extracts was determined by comparing with activities of substances such as luteolin, quercetin, BHA, BHT and sylimarin (reference substances), which possess some antioxidant potential. In Table 1. FRSA values obtained for plant extracts together with reference substances in DPPH assay are shown. Various extracts showed different activity in this assay. Methanol extract derived from stems and leaves showed the highest inhibitory activity with IC_{50} of 6 mg mL^{-1}. The lowest DPPH scavenging activity was shown by ethyl acetate extract derived from flowers (IC_{50} 11 mg mL^{-1}). The other *Helichrysum* extracts demonstrated similar DPPH scavenging activity (8-9 mg mL^{-1}). When compared to the reference substances, the *Helichrysum* extracts were found to be less efficient in radical scavenging. Luteolin, quercetin, BHA, and sylimarin interacted intensively with DPPH (IC_{50}: 0.09, 0.06, 0.152 and 1.96 mg mL^{-1}, respectively) while BHT possessed moderate scavenging properties (3.52 mg mL^{-1}). The scavenging effects can probably be attributed to the flavonoids present in the examined extracts, but could also be the result of the activity of other secondary metabolites, such as volatile oils, carotenoids, and vitamins, that in the case may contributed to the antioxidant capacity (16,17).

Table 1. DPPH radical scavenging activity of the *Helichrysum plicatum* extracts against luteolin, quercetin, BHA, BHT and sylimarin as standards.

	IC_{50}[a] (mg ml^{-1})
Flower extracts	
Methanol	9 ± 0.7[b]
Etanol:water	11 ± 2.2
After hydrolysis	8 ± 1.9
Stem and leaf extracts	
Methanol	6 ± 3.0
Etanol:water	8 ± 0.5
After hydrolysis	8 ± 2.4
Reference substances	
Luteolin	0.09 ± 0.01
Quercetin	0.06 ± 0.02
Sylimarin	1.96 ± 0.03
BHA	0.152 ± 0.002
BHT	3.52 ± 0.02

[a] IC_{50} denotes the extract concentration at which the absorbance shows 50 % of control
[b] All values are shown as the mean ± SD of more than 3 measurements

Hydroxyl radical scavenging activity

The Fenton reaction describes the oxidation of H_2O_2 by Fe^{2+} to $OH^{\bullet}$ and Fe^{3+}. In the model employed in this experiment, the production of $OH^{\bullet}$ induced oxidation of the deoxyribose, which in turn reacted with TBA to produce a TBA reactive chromofore that was detectable at 535 nm, thus enabling assessment of antioxidant activity of plant extracts (10).

Fig. 1 presents the results of the effects of examined *Helichrysum* extracts, reference substances (luteolin, quercetin, BHA, BHT and sylimarin) as well as control solution on $OH^{\bullet}$ radical production. The results show that all extracts of *Helichrysum plicatum* inhibited the production of $OH^{\bullet}$ radicals. The strongest inhibitory activity was exhibited by the H-extract derived from stems and leaves (58%). When compared to the reference substances, luteolin, quercetin, BHA, BHT and sylimarin (48%, 42%, 52%, 47% and 43%, respectively), the extracts had slightly lower activity, except of H-extract derived from stems and leaves with greater activity than BHT.

The findings demonstrate hydroxyl radical scavenging potential of *Helichrysum* extracts against Fenton reaction induced $OH^{\bullet}$ generation, showing similar performance with luteolin, quercetin, BHA, BHT and sylimarin. This activity is mainly due to the redox properties of flavonoids, which

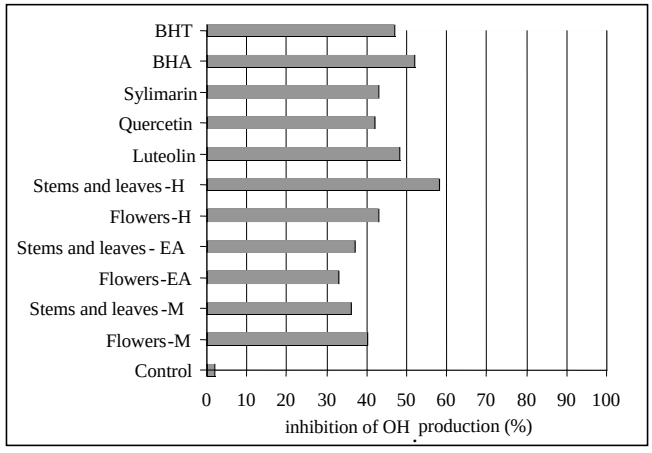

Fig. 1. Effects of *Helichrysum plicatum* extracts against luteolin, quercetin, BHA, BHT, sylimarin as standards and control on the *in vitro* $OH^{\bullet}$ production.

allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have a metal chelating potential (22). The slight quantitative differences in the amounts of flavonoids (2) might also explain the minor differences between the activities of the extracts.

Antioxidant activity

For the screening of antioxidant potential of *Helichrysum* extracts, β-carotene/linoleate model system has been applied (11). This test involves a reaction between a potential antioxidant, β-carotene and linoleic acid. β-Carotene undergoes rapid discoloration in the absence of an antioxidant. The presence of antioxidant compounds can hinter the extent of β-carotene destruction by neutralizing the linoleate free radical and any other free radicals formed within the system.

The antioxidant activity of each extract of *Helichrysum plicatum*, BHA and control is presented in Fig. 2. According to the preventive activity against bleaching of β-carotene, M-extract (46%) derived from flowers and H-extract (44%) from stems and leaves are the most promising

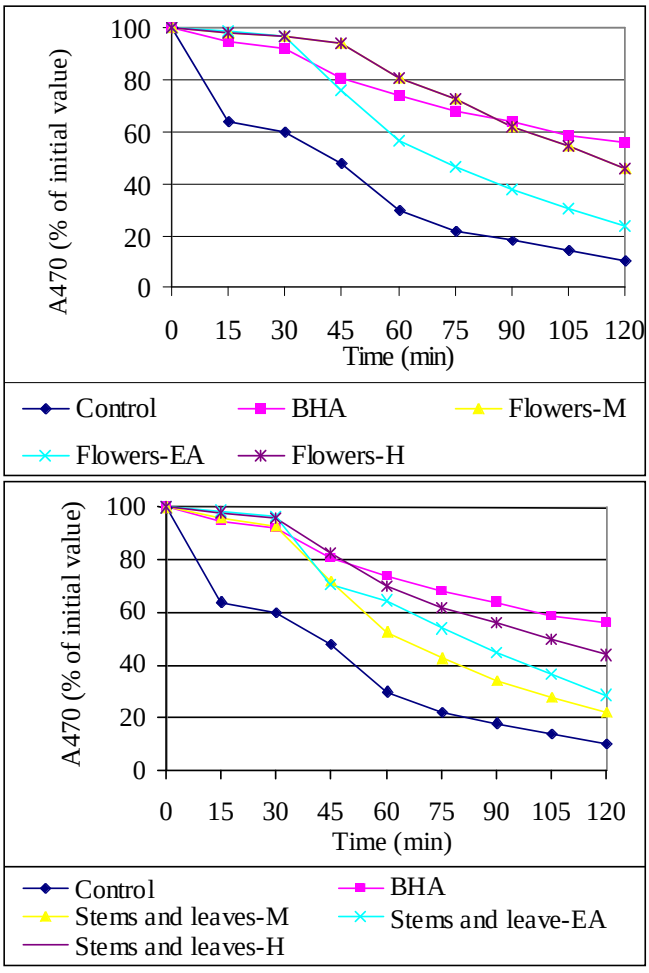

Fig. 2. Effect of *Helichrysum plicatum* extracts and control on oxidation of the β-carotene-linoleic acid model system.

Helichrysum extracts. M-extract (22%) and EA-extract (29%) derived from stems and leaves showed short and weak inhibition of bleaching, close to that of flowers EA-extract (24%). H-extract (15%) from flowers was found to be less efficient in this model system. The inhibitory effect on β-carotene bleaching of the all *Helichrysum* extracts was lower than that of BHA.

The antioxidant activity of *Helichrysum plicatum* extracts has been attributed to various mechanisms, among which is neutralizing β-carotene destruction. Also, we can suggest that there is always no linear correlation between antioxidant activity and the content of flavonoids (2). This indicates that the concentration of flavonoids is not the only factor related to the antioxidant activity. Possible synergism of flavonoids with other components present in the extracts may be responsible for this observation.

Conclusion

Results of this study suggested that *Helichrysum plicatum* DC. could be regarded as a good source for natural antioxidant. Its extracts exhibit potent free radical scavenging, hydroxyl radical scavenging and antioxidant activity *in vitro*. The information from this study can explain the traditional use and the further development of these extracts into new pharmaceuticals.

The data reported here can be consider as the first information on the antioxidant properties of Macedonian *Helichrysum plicatum*. Further studies are needed to evaluate the *in vivo* potential of these extracts in animal models.

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Резиме

Антиоксидативен потенцијал на *Helichrysum plicatum* DC. (Asteraceae)

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Клучни зборови: *Helichrysum plicatum*, флавоноиди, DPPH, хидроксил радикал, липидна пероксидација.

Опишана е способноста на различни екстракти од цветови, стебла и листови на *H. plicatum* да делуваат како природни антиоксиданси во различни *in vitro* експериментални модели во кои се вклучени реакции со слободни радикали: инхибиција на DPPH (1,1-diphenyl-2-picrylhydrazyl) радикалот, инхибиција на хидроксил радикали и заштита на β-каротен-линоленска киселина модел системот. Испитуваните екстракти покажуваат активност за фаќање на слободни радикали со IC_{50} од 6 до 11 mg/ml. Екстрактите се способни да реагираат со OH• радикалот со инхибиција на продукција меѓу 33-58%. Изразена превентивна активост покажуваат и кон промените на β-каротенот (15-40% од почетната вредност, после 120 минути). Антиоксидативната активност на екстрактите во експерименталните системи е споредувана со референтните супстанции: лутеолин, кверцетин, BHA, BHT и силимарин (главниот активен принцип на растението млечен трн - *Silybum marianum* L.*)*. Резултатите од испитувањето укажуваат дека *Helichrysum plicatum* претставува природен извор со антиоксидативен потенцијал.

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Original scientific paper

Serum Cistatin C in patients with delayed graft function

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Abstract

Despite recent studies showing that serum Cystatin C(CysC) is a better marker for glomerular filtration rate (GFR) than the ubiquitously used creatinine, the clinical utility of this remains to be evaluated. This marker is very sensitive for alograft function after renal transplantation. Concentration of CysC was compared with that of creatinine. Decreased renal function was followed in 64 transplanted patients. Plasma CysC significantly correlated ($r=0.625$, $p<0.001$) with creatinine in healthy controls. In these patients the mean plasma creatinine and Cystatin C concentrations were: 81+/-13 mmol/L,0.90 +/-0.22 mg/L, respectively. Plasma Cystatin C and creatinine significantly correlated throughout the post-transplantation period ($r=0.686$, $p<0.001$), but we confirmed differences between kinetics of these parameters. In the first four days after transplantation the CysC concentration was normalized faster than creatinine concentration. Development of acute rejection episode (between 5 and 7 days) showed high sensitivity and specificity of the changes of CysC compared with those of creatinine.

Key words: renal transplantation, delayed graft function, cystatin C

Introduction

Cystatin C (Cys C) is a nonglycosylated basic protein (13.36 kDa) and can be found in a variety of biologic fluids (1). CysC serum concentration is not influenced by gender, inflammation, or lean tissue mass and is regarded to be mainly determined by glomerular filtration rate (GFR) (2, 3). Cystatin C has been described as meeting many of the characteristics of an ideal GFR marker (e.g., endogenously produced at a constant rate, freely filtered in the glomerulus, neither reabsorbed nor secreted in the renal tubule, not extrarenally eliminated) and has been reported to be at least as accurate as the commonly used serum creatinine to detect impaired renal function in various patient groups, including renal transplant patients (4-11).

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In recent literature, cystatin C has been advocated as a new and more accurate estimate of GFR (1).Cystatin C is a 13-kDa endogenous cysteine proteinase inhibitor produced by all nucleated cells at a constant rate and broken down completely in the renal tubuli (12). Cystatin C concentrations are independent of age and body weight, and there is no need for urine collection for clearance estimations. Furthermore, serum concentrations of cystatin C are not influenced by malignancy or inflammation. In contrast, the often-used serum creatinine concentration is supposedly influenced by dietary intake, renal tubular metabolism, age, and variations in muscle mass. There are also various analytical difficulties with the widely used Jaffe colorimetric assay for creatinine. A slight decrease in GFR has been found in patients with hypothyroidism, which improved significantly after treatment (2,3). We wondered whether cystatin C would also be a good marker of renal function in case of thyroid dysfunction. Thyroid hormones have metabolic effects and thus, thyroid state could influence plasma cystatin C concentrations.

Patients are at risk of acute damage of the transplanted kidney because of rejection or toxicity from immunosuppressant therapy. Earlier detection of renal damage may lead to more effective intervention. In a preliminary study, LeBricon et al. (13) first suggested that CysC was more sensitive than serum creatinine(SCr) for detecting decreases in GFR and delayed graft function in renal transplant patients. As in most studies, plasma CysC measurements correlated well with SCr and creatinine clearence (CrCl). However, in the three cases of acute renal rejection that were confirmed by biopsy, the increase in plasma CysC values was more pronounced than that observed for SCr.

After renal transplantation, plasma (or serum) creatinine is the most common marker for assessment of allograft function. In a steady-state muscular mass balance, the plasma creatinine concentration is assumed to reflect glomerular filtration rate (GFR) (14). However, plasma creatinine is far from being an ideal marker of GFR, despite its convenience and low cost (15) Plasma creatinine suffers a high degree of interindividual variability related to sex, age, body composition, and dietary factors (16). With altered renal function, the plasma creatinine concentration increases only when the GFR is reduced by >50%. Furthermore, secretion or reabsorption of creatinine by the renal tubule is highly unpredictable, thus leading to under- or overestimation of GFR (15).

Numerous drugs and endogenous substances also interfere with the measurement of creatinine by the Jaffé technique or enzymatic methods, leading to falsely high or low creatinine values (17).

Experimental

Samples

Sixty-four patients with end-stage renal disease undergoing renal transplantation were included in this study. Primary diagnosis of the investigated patients was: chronic interstitial nephropathy (n = 8), diabetic glomerulopathy (n = 12), polycystic kidney disease (n = 3), nephrosclerosis (n = 3), focal segmental glomerulosclerosis (n = 10), IgA nephropathy (n = 8), membranous glomerulonephritis (n = 11), mesangiocapillary glomerulonephritis (n = 1), and unknown (n = 4). Immunosuppressive regimen included steroids (methylprednisolone at the initial dose of 500 mg, followed by 1 mg · kg-1 · day-1, progressively tapered) and cyclosporine A (initial dose of 8 mg · kg-1 · day-1, and then adjusted according to blood concentrations) or FK506 in cases of cyclosporine intolerance (at the dose of 0.1 mg · kg-1 · day-1). The patients were classified indo two groups according to the clinical diagnosis: with or without delayed graft function.

The control group consisted of 50 healthy persons. They were free of cardiac, liver or renal diseases or hypertension and had normal urine analysis and normal sera urea,creatinine and Cys C concentration.

Methods

Delayed graft function (DGF) was defined as a requirement for dialysis during the first 2 weeks after transplantation. All patients were on conventional dialysis. Episodes of acute rejection diagnosed by renal biopsy were treated with 5 days of intravenous methylprednisolone. This study was in accordance with the ethics standards of the Helsinki Declaration of 1975, revised in 1983.

Allograft function was evaluated on a daily basis starting on the day of surgery (day 0) and for 25 days thereafter or until hospital discharge, whichever occurred first. Blood (7 mL) was drawn by venipuncture in a Vacutainer® Tube (Becton Dickinson) before centrifugation (3500g at 20 °C for 15 min) and analyzed for creatinine and CysC. Creatinine concentration was enzymatically assayed on a INTEGRA biochemical analyzer. The sample volume was 10 µL, and the assay was performed at 37°C; total analysis time was 7 min. The interassay imprecision (CV; n = 50) was <3%.

The concentration of CysC was measured using a DAKO Immunoturbidimetric assay on a Cobas Mira biochemical analyser.The reference serum interval was 0.80 – 1.25 mg/L. Briefly, the assay is performed at room temperature with a six-point calibration covering the range of 0.23–7.25 mg/L. The calibrator used is a purified Cys C from human urine (1.45 mg/L). The sample volume is 80 µL. The time for analysis is 6 min, each subsequent sample reading being available after 8 s. The interassay CV (n = 20) was <4% for both the low (1.4 mg/L) and high (4.2 mg/L) controls.

Calculations

The percentage of discordant changes in plasma cystatin C and creatinine concentrations was calculated on a day-to-day basis after transplantation. A change in opposite direction (increase/decrease) of >10% between the two markers was considered as discordant. On hospital discharge or at the end of the 25 days period, creatinine clearance was estimated from plasma creatinine using the formula of Cockcroft and Gault (14). A cutoff of 80 mL/min was selected for normal estimated creatinine clearance (25).

Statistical analysis

Data are presented as mean ± SD or as median and range when appropriate after checking for gaussian distribution. Differences between two groups were evaluated

by the Wilcoxon's signed-rank test. Multiple comparisons were performed by the Friedman's repeated-measure ANOVA on ranks followed by the Dunn's test. Correlation between techniques was evaluated by linear regression and ANOVA. Results with P <0.05 were considered statistically significant.

Results and discussion

CysC and creatinine concentration were measured in 50 healthy adults (39 +/- 9 years). Data were analyzed by linear regression and ANOVA (P < 0.05 considered significant).The concentration of Cystatin C significantly correlated (r = 0.625; P<0.001) with creatinine (Fig. 1). Cystatin C serum concentration was not influenced by age (r=0.195; not significant). The calculated reference interval for creatinine was 58-109 mmol/L (males and females) and the cystatin C : 0.80 – 1.25 mg/L.

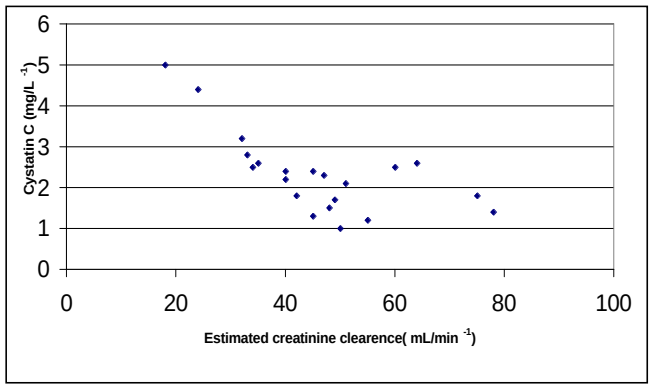

Fig. 1. Relationship between serum Cystatin C and serum creatinine in healthy controls (r= 0.625, p < 0.001)

Transplant patients were separated into two groups: normal course (absent of complications; n= 38) (Fig. 2); DGF and acute rejection episode defined as requiring hemodialysis during the first 2 weeks after surgery (n=26).

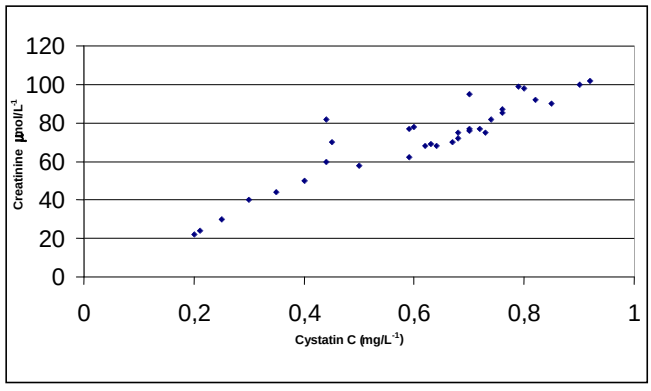

Fig. 2. Serum Cystatin C and serum creatinine in renal transplant patients without DGF

In patients without DGF a significant decline in plasma concentration was more rapidly obtained for cystatin C that for creatinine.

Starting on day 4 post-transplantation the decrease in plasma concentration in patients without DGF was more pronounced for creatinine that for cystatin C (Fig. 3).

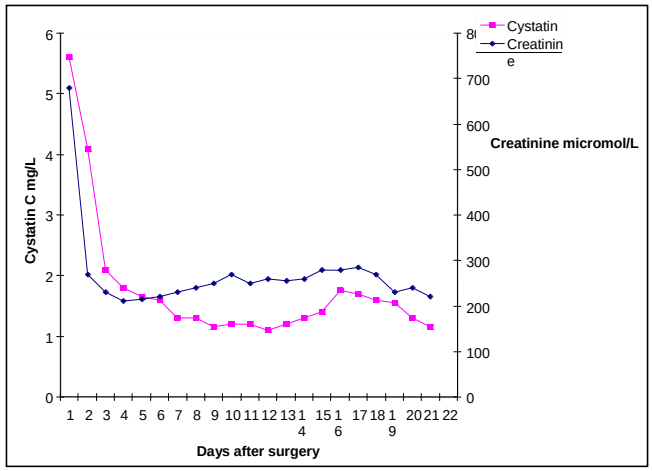

Fig. 3. Serum Cystatin C and serum creatinine in renal transplant patients with DGF

In patients with DGF, the reduction in plasma concentration was not significant until day 14 for creatnine and day 17 for cystatin C.

At the end of the 25 days - study period, a 50% reduction from the initial plasma creatinine concentration was observed in patients with DGF vs 35% for Cystatin C.The frequency of discordant daily changes in cystatin C and creatinine values was significantly higher in patients with DGF(40%) than in those without DGF (21%,P < 0.01).

In cases with acute rejection episode was demonstrated by persistent increases in both plasma creatinine and cystatin C concentration. Serum cystatin C gradually increase (115%) during 7 days before diagnosis (nephrotoxicity – FK506-regressed spontaneously).

At the end of the study (day 25), serum creatinine was 150 µmol/L (79–602 µmol/L) vs 2.12 mg/L (1.04–5.54 mg/L) for cystatin C. Both markers significantly correlated (r = 0.812; P <0.001). Estimated creatinine clearance by the Cockcroft and Gault formula (14) in patients with stable renal function (no hemodialysis during the last week, n = 19) was 49 mL/min (15–66 mL/min), and no patient was within the reference interval (>80 mL/min). Serum creatinine was within the reference interval for three patients (80-109 µmol/L) compared with none for cystatin C. The relationship between estimated creatinine clearance and the serum concentrations of creatinine and cystatin C in patients with stable renal function (n = 22) is presented in Fig. 4 and 5.

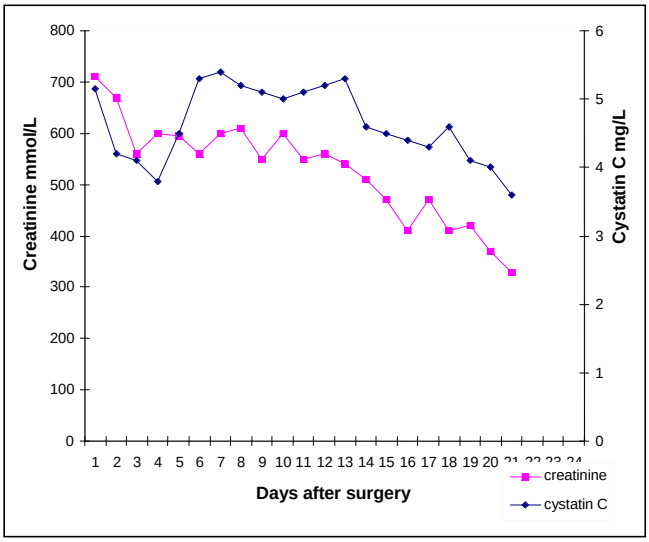

Fig. 4. Relationship between serum Cystatin C and GFR

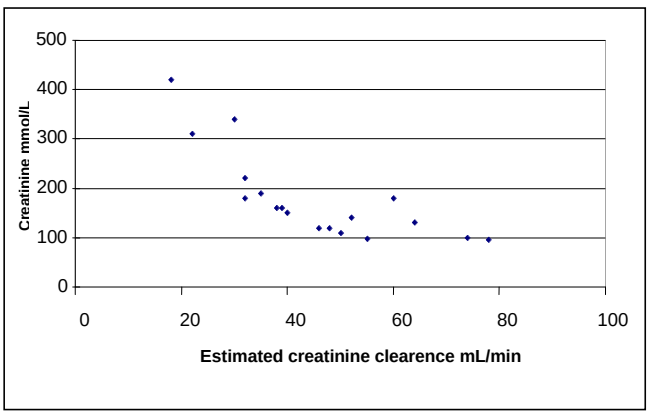

Fig. 5. Relationship between serum creatinine and GFR

There was a significant correlation between estimated creatinine clearance and serum cystatin C ($r = 0.714$; $P < 0.001$).

Discusion

Sensitive and reliable recognition of changes in GFR is of primary importance in transplant patients. A DGF is an identified and independent risk factor for graft survival (26) Acute rejection is also an established risk factor for renal graft failure, defined as a return of patient to hemodialysis (18-20). Recent studies have suggested that cystatin C might be a potential better marker of GFR than plasma creatinine such as in renal transplant patients (28-30).

The mean cystatin C plasma concentration in our group of healthy adults was similar to that reported in a group of healthy subjects (n = 50; mean age, 40 years), using an immunoturbidimetric assay (0.65 ± 0.05 mg/L) (30). Slightly higher values (mean cystatin C, 0.80 mg/L) were reported by others (27) in a group of 52 adults with normal renal function (ages, 21–79 years). These data are in agreement with an increase in plasma cystatin C values with age, especially after the age of 50 years (35). We found no the sex difference reported previously (22,23). Serum cystatin C correlated poorly with serum creatinine, as reported previously in subjects with GFR >80 mL/min (24) or GFR >70 $mL \cdot min^{-1} \cdot 1.73\ m^{-2}$ (23). These data suggest that different physiological factors (such as sex, dietary factors, or body composition) influence cystatin C and creatinine plasma concentrations in healthy adults.

In renal transplant patients, plasma cystatin C concentrations paralleled those of creatinine regardless of graft function (absence or presence of DGF). Consequently, serum cystatin C and creatinine significantly correlated over the postoperative study period as observed previously in adult renal transplant patients (31) and subjects suffering from chronic renal disease (13). Some differences, however, were apparent in their respective plasma kinetics. During the first 4 days post-transplantation, the cystatin C serum concentration decreased more rapidly than that of creatinine. Tanstubular leakage of the low-molecular weight (M_r 100) creatinine (23) has been reported in acute renal failure, thus leading to high plasma creatinine values. Starting on day 4 post-transplantation, decrease in serum concentration became more prominent for creatinine than for cystatin C, which might be attributable to an underestimation of GFR by plasma cystatin C, an overestimation of GFR by plasma creatinine, or both. A stronger correlation between cystatin C than between creatinine and the measured GFR has been reported in adults suffering from renal diseases (18).

For patient follow-up, the ability to detect rapid changes in GFR is clinically more important than accuracy itself. With diminished GFR, a significant increase in plasma concentration of cystatin C and creatinine will depend on the rate of its accumulation in plasma, which depends on its production rate and distribution volume, but also on its biological intraindividual variation. Repeated measures obtained in healthy subjects (30) suggested that intraindividual variation might be more important for cystatin C (13.3%) than for creatinine (4.9%). If true, cystatin C would be less sensitive for the detection of acute rejection episodes for a given individual than creatinine (30). In all episodes of acute rejection and acute nephrotoxicity in our study, the plasma cystatin C concentration broadly paralleled that of creatinine. The rise in plasma cystatin C concentration was more prominent than in creatinine.

After renal transplantation, hemodialysis was required in almost 55% of our patients. We found a higher degree of discrepancy (45% of discordant results; $P < 0.01$) between cystatin C and creatinine kinetics in patients requiring hemodialysis than in those with a normal course (19%). In addi-

tion, cystatin C and creatinine weakly correlated in hemodialyzed patients (r = 0.429). The molecular weight of cystatin C is 13 300 with an Einstein-Stokes radius of 30–40 Å, which is much higher than creatinine (M_r 100 and 3 Å) (15). In a large study of 112 patients on stable maintenance hemodialysis, a 30% reduction in serum cystatin C was observed after dialysis with mostly AN69 high-flux membranes (23). As expected, the elimination of cystatin C during dialysis increased with the ultrafiltration coefficient (UFC) of the membrane, an estimate of the permeability: 0% (vs 40% for creatinine) for UFC <15 mL $\cdot$ h^{-1} $\cdot$ m^2 $\cdot$ mmHg and ~ 60% (as for creatinine) for UFC >15 mL $\cdot$ h^{-1} $\cdot$ m^2 $\cdot$ mmHg (23). Thus, removal of cystatin C by hemodialysis seems highly dependent on the type of membrane selected. This is an important issue in renal transplantation because it could limit the use of plasma cystatin C as a marker of graft function in patients with DGF. On the other hand, if poorly filtered by dialysis membranes, plasma cystatin C could be used by the nephrologist to monitor appropriate duration of hemodialysis in patients with DGF. Prolonged unnecessary hemodialysis could be avoided as soon as a significant decrease in plasma cystatin C is obtained by dialysis.

In conclusion, the determination of cystatin C concentration is an alternative and more accurate marker of GFR than creatinine in adult transplantation. In some cases, a more prominent rise in serum cystatin C values allows a more rapid diagnosis of acute rejection or treatment nephrotoxicity. Further prospective studies are needed to evaluate this last issue and the potential of plasma cystatin C in the long term follow-up of graft function in renal transplantation.

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Резиме

Серум Cistatin C кај пациенти со нарушена графт функција

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Клучни зборови: ренална трансплантација, нарушена графт функција, Cystatin C

Изработената студија укажува дека серумската концентрација на Cystatin C (Cys C) е подобар маркер за GFR во споредба со стандардниот креатинин, што беше и клинички евалиурано. Овој маркер е многу поосетлив за графт функцијата после бубрежна трансплантација. Концентрацијата на Cys C е споредена со онаа на креатининот. Намалувањето на бубрежната функција е следено кај 64 трансплантирани пациенти. Концентрацијата на Cys C сигнификантно корелира (r=0.625, p<0.001) со креатининот кај контролната група. Кај овие пациенти средните креатинин и цистатин Ц концентрации се: 81+/-13 mmol/L односно 0.90 +/-0.22 mg/L. Плазма концентрациите на цистатин Ц и креатининот сигнификантно корелираат во пост-трансплантациониот период (r=0.686, p<0.001), но потврдивме разлики во кинетиката на овие параметри. Во првите четири дена после трансплантација Cys C се нормализира побрзо од таа на креатининот. Развитокот на акутна реакција (помеѓу 5 и 7 ден) укажува на високата осетливост и специфичност на промените на Cys C споредени со тие на креатининот.

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Original scientific paper

Comparative investigation of the sweet and bitter orange essential oil (*Citrus sinensis* and *Citrus aurantium*)

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Abstract

The volatile fraction composition of commercially produced sweet and bitter orange oil from fruit peels was studied using GC-MS. More than fifty components were identified in the oils using their mass spectra and linear retention indices. The monoterpene limonene was the most abundant component even though not in a quantity expected for a fresh orange essential oil. Aldehydes, followed by alcohols and esters, were the main components in the oxygenated fraction. Aldehydes were the major oxygenated components in the sweet orange oil, whereas alcohols and esters were present in higher amounts in the bitter orange oil. Among them, nonanal, decanal and linalool are the most important components for the flavour of sweet orange oil and carvon is the most important ketone for the flavour of bitter orange oil in combination with the other components. The amount of carvon gives a good indication about the freshness of the oil and the quantities of α-pinene and β-pinene, sabinene and myrcene give an indication about the natural or artificially changed composition of the essential oils.

Key words: *Citrus sinensis*, *Citrus aurantium*, peel oil, GC-MS, volatile components

Introduction

Cold pressed orange oil is widely used in beverage, perfumery industry and aromatherapy. In general, there are two types of orange oils: sweet orange oil from *Citrus sinensis* and bitter orange oil from *Citrus aurantium*.

Sweet orange oil is the most widely used citrus oil. It possesses a light sweet, fresh top note with fruity and aldehydic character (1). It is widely used in the flavour industry especially in beverages and candies. It can provide the top note for citrus flavouring as well as characteristic and most universally accepted flavour. The sweetness and in the same time the refreshing note makes them appropriate base for still and carbonated soft drinks.

Most of the orange oils on the world market are Italian and American industrial oils, but there are also oils produced in Spain, and in the South American countries. Moreover, studies on laboratory-extracted oils from the peel of fruits of special cultivars from different regions such as Algeria, Libya, Israel, Russia, China and Japan have been published (1).

Bitter orange oil possesses fresh citrus top notes, but is considered less sweet, and even bitter and dry. It has floral and aldehydic characteristics (1). The tenacity is greater than the most other citrus oils. In flavourings, this oil can be used to provide a citrus top note or act as a modifier in the citrus blend. The major application is in the citrus flavourings for beverages, especially liqueurs. It also intensifies the orange character in soft drinks.

Most of the papers pertinent to sweet orange oil composition were reviewed by Sawamura (2, 3) and Lawrence (4). A large number of papers deal with the composition of the volatile fraction of sweet orange oil and in many of them its differences in relation to the cultivars are reported with particular references to the different content of aliphatic aldehydes and linalool (5-10), and the volatile components of bitter orange oil (11,12).

The goal of this work is to present a way of comparative investigation of the cold pressed sweet and bitter

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orange oils found in the market and to see the difference between the oils. The results are compared with the published ones for fresh orange oils and some indications about the freshness and the origin are discussed.

Experimental

Two commercial samples of oils produced from peel of *Citrus sinensis* and *Citrus aurantium* were kindly supplied from an Italian flavour company.

Each sample was analysed by GC/MS using a HP 6890 Gas chromatograph equipped with a HP 5973 mass selective detector with a HP-5 fused silica column (30 m x 0.25mm, 0.25 μm film thickness). The column temperature was changed linearly from 40 to 180 °C by 4 °C/min, and after that 20°C/min to 260°C. 0.2 μL were injected at a split ratio of 1:100. The inlet temperature was 260°C and the transfer line temperature was 280°C.

The MS library used was Wiley 275. Also, compound identification was checked by linear retention indices with standard series of alkanes (C_8-C_{32}) and compared with the LRI values in the database of the Citrus Research and Education Centre at the University of Florida (13) and in the book of Adams (14).

Results and discussion

Sample preparation is one of the most important processes in flavour research, because the aroma compounds are substantially volatile and unstable against heat (3). GC-MS is a valuable tool in characterization of essential oils because the interpretation of the results of qualitative and quantitative analysis can enable an insight in the process of production, storage and age of the commercially produced oils.

In this work, more than fifty components were identified in the analysed sweet and bitter orange oils. The identified compounds and their linear retention indices calculated on HP-5 column are given in Table 1. For each sample, the quantitative composition (as a relative percentage of peak area) for each component is given together with the aroma descriptors for the components as found in the database of the Citrus Research and Education Centre at the University of Florida (13) and by Choi (15).

The GC-MS chromatograms obtained for both samples of essential oils of sweet and bitter orange oil are given in Fig. 1 and Fig. 2, respectively.

The data in Table 1 show that different quantities and ratios of the same components give different taste of the aroma. According to the data, the quantities of α-pinene,

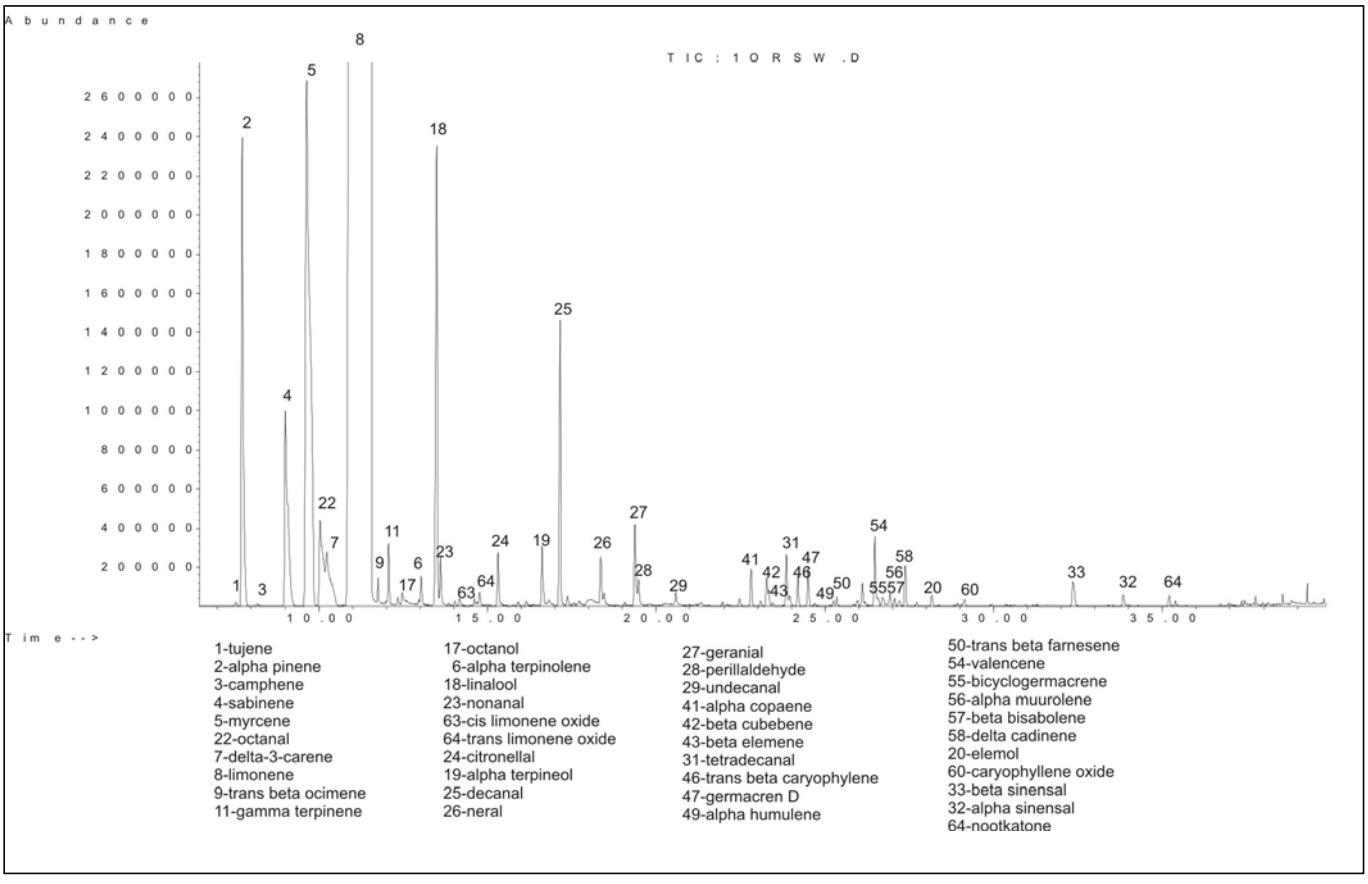
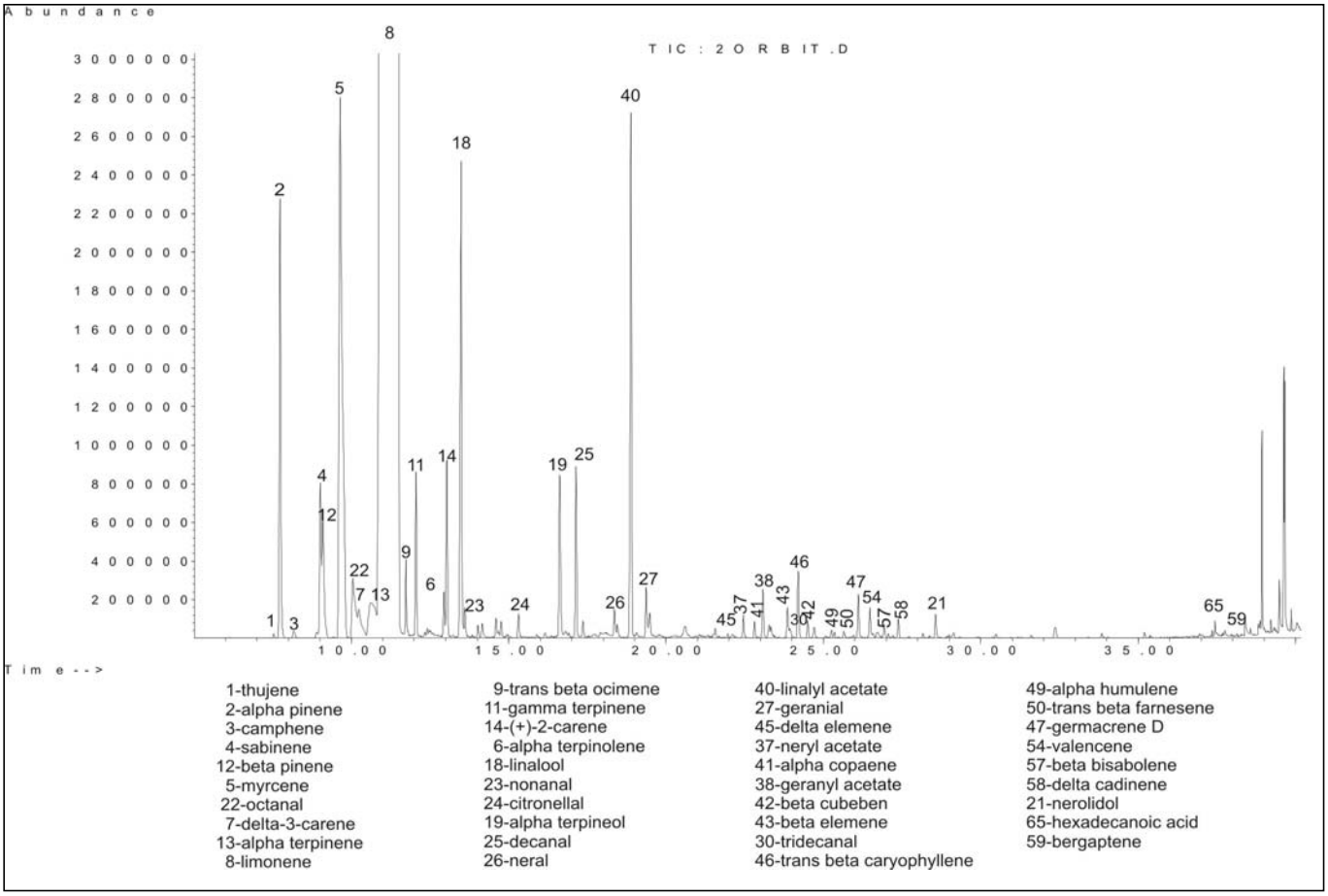


Fig. 1. Chromatogram of sweet orange peel oil



1-thujene
2-alpha pinene
3-camphene
4-sabinene
12-beta pinene
5-myrcene
22-octanal
7-delta-3-carene
13-alpha terpinene
8-limonene

9-trans beta ocimene
11-gamma terpinene
14-(+)-2-carene
6-alpha terpinolene
18-linalool
23-nonanal
24-citronellal
19-alpha terpineol
25-decanal
26-neral

40-linalyl acetate
27-geranial
45-delta elemene
37-neryl acetate
41-alpha copaene
38-geranyl acetate
42-beta cubeben
43-beta elemene
30-tridecanal
46-trans beta caryophyllene

49-alpha humulene
50-trans beta farnesene
47-germacrene D
54-valencene
57-beta bisabolene
58-delta cadinene
21-nerolidol
65-hexadecanoic acid
59-bergaptene

Fig. 2. Chromatogram of bitter orange peel oil

camphene, myrcene and octanal are higher than the usual values for cold-pressed sweet and bitter orange oil (1). On the other hand, the quantities of limonene are lower (76.65 % in sweet orange oil and 71.2 % in bitter orange oil) than expected according to the published data for fresh sweet and bitter orange essential oils (over 90 %) (1).

The quantity of α-terpinolene which gives the citrus and pine note according to odor description database of Citrus Research and Education Centre (13) is almost six times greater in bitter orange oil than in sweet orange oil. The quantity of valencene in sweet orange oil is more than double compared to bitter orange oil, which is expected because this compound gives the specific sweet citrus odor for sweet orange.

Aldehydes are the most important compounds for the citrus flavour. The quantity of aldehydes in sweet orange peel oil is double compared to the aldehydes content in the bitter orange oil. Octanal, nonanal and decanal, which are very active odor compounds and have a sweet note, are in higher quantity in sweet than in bitter orange oil (2).

On the other hand, alcohols content in bitter orange oil is higher than in sweet orange oil, but linaool in both analysed commercial oils is in bigger quantity than expected from the published data (1).

Also the quantity of esters is three times higher in bitter orange oil in comparison to quantity of esters in sweet orange oil, because the quantity of geranyl acetate and linalyl acetate are very high in bitter orange oil.

There are some components expected and identified in the in bitter orange oil like +-2-carene, n-octyl acetate, linalyl acetate, 2,4-decadienal, δ-elemene, germacren B, nerolidol, bergapten, which are missed in the sweet orange oil. β-pinene is also missed in sweet orange oil and it is present in bitter orange oil in enough high quantity.

It is well known that the bitter taste of bitter orange oil predominantly comes from the nonvolatile components of the peel, but also the volatile components give a significant part of the aroma of bitter orange oil (1). The component which gives a bitter odor, carvon, is in higher quantity in bitter orange than in sweet orange oil. It has been observed that orange oil deteriorates very rapidly in aqueous acidic environment and under the influence of light and oxygen. Relatively high quantity of carvon in both oils has been identified as the major degradation product (Fig. 3), and has also been reported as a constituent of various old citrus oils (11, 16). From this we can include that the oils purchased from the market are not fresh.

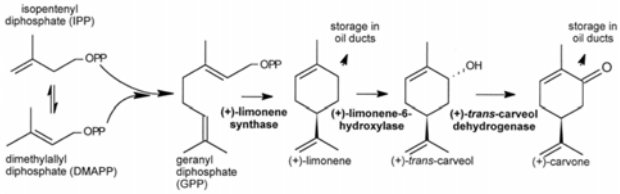


Fig. 3. Biosynhesis of limonene and its transformation to carvone during storage

All these results indicate that the two commercially produced orange oils purchased from the flavour market are not fresh and original cold pressed essential oils, but some of the components, like limonene, are removed from the oil and some of the components, like α-pinene, sabinene are added to the oils by physical or chemical methods (1).

In conclusion, the qualitative and quantitative analysis of the commercial bitter orange oil shows that this oil is not pure cold pressed oil because the ratio α-pinene/β-pinene is greater then 0.8 which indicates addition of grapefruit terpenes in the bitter orange oil (1). On the other hand, the significant amount of myrcene (>2 %) and octanal (>0.2 %) in the commercial sweet orange oil gives an indication that the oil is not an original cold pressed oil, but has been modified in the manufacturing process, which is also supported by the significantly reduced content of limonene.

Table 1. Percentage composition as single components for the two different orange oils, the LRI index for each component of HP-5 column and aroma descriptors

	Component	LRI	% in C. sinensis	% in C. aurantium	Aroma descriptor [13, 15]
	Monoterpenes				
1.	á-tuje ne	926	0.02	0.02	
2.	á-pinene	932	2.57	2.10	pine-like, resinous, green, sweet
3.	camphene	947	0.01	0.06	warm, oily, camporaceous
4.	sabinene	972	1.83	0.84	warm, oily, peppery, green
5.	myrcene	992	6.90	5.86	musty, wet soil
6.	á-terpinolene	1090	0.13	0.77	citrus, pine
7.	ä-3-carene	1010	0.79	0.37	sweet
8.	limonene	1033	76.65	71.2	citrus-like, fresh
9.	trans-â-ocimene	1054	0.10	0.32	herbacous, flowery, sweet, warm
10.	â-phellandrene	1060	0.01	-	citrus-like, fresh
11.	ã-terpinene	1062	0.23	0.67	lemony, lime-like
12.	â-pinene	975		0.79	resinous, dry, woody
13.	á-terpinene	1021		0.05	lemony, citrusy
14.	+-2-carene	1088		0.20	
15.	ä-4-carene	1351		0.02	
	Total		89.24	83.27	
	hydrates				
16.	cis-sabinene hydrate	1070	0.03	0.04	
	Total		0.03	0.04	
	alcohols				
17.	octanol	1074	0.07	0.11	soapy
18.	linalool	1103	2.30	2.50	floral, green, citrus
19.	á-terpineol	1192	0.31	0.83	floral, lilac-like
18.	nerol	1233	0.07	-	fruity, floral
20.	elemol	1552	0.06	0.02	sweet, woody, faint
21.	nerolidol	1566		0.12	woody, floral, mild
	Total		2.81	3.58	
	aldehydes				
22.	octanal	1004	0.96	0.67	fatty, tallowy, citrus-like
23.	nonanal	1107	0.22	0.17	piney, floral, citrusy
24.	citronellal	1155	0.25	0.12	powerful, floral, lemon
25.	decanal	1208	1.27	0.78	beefy, musty, marine, cucumber

26.	neral	1243	0.24	0.15	lemony, citrusy
27.	geranial	1273	0.42	0.27	citrus-like, flowery, fruity
28.	perillaldehyde	1276	0.14	-	
29.	undecanal	1309	0.08	-	pleasant waxy, floral
30.	tridecanal	1410	0.24	0.15	waxy, fresh, citrusy, powerful
31.	tetradecanal	1614	0.01	-	fresh, herbaceous
32.	á-sinensal	1757	0.06	0.02	orange-like
33.	â-sinensal	1700	0.17	0.08	orange peel
34.	2,4-decadienal	1319		0.02	geranium, powerful
35.	3-dodecen-1-al	1468		0.04	
	Total		4.06	2.47	
	esters				
36.	citronellyl acetate	1356	0.01	-	fresh, rosy, fruity
37.	neryl acetate	1367	0.02	0.12	fruity, floral, very sweet
38.	geranyl acetate	1386	0.02	0.22	dry, herbaceous
39.	n-octyl acetate	1214		0.09	fruity, slightly fatty
40.	linalyl acetate	1259		2.54	floral-fruity
	Total		0.05	2.97	
	sesquiterpenes				
41.	á-copaene	1377	0.16	0.08	
42.	â-cubebene	1392	0.18	0.07	fruity, green
43	â-elemene	1394	0.07	0.06	fruity
44.	aromadendrene	1397	0.02	-	
45.	ä-elemene	1339		0.05	
46.	trans-â-caryophyllene	1421	0.14	0.32	citrus-like, fresh
47.	germacrene-D	1483	0.11	0.22	
48.	trans-á-bergamotene	1437	0.01	0.06	
49.	á-humulene	1455	0.03	0.04	woody
50.	trans-â-farnesene	1458	0.04	0.03	sweet, fruity
51.	á-amorphene	1478	0.03	0.02	
52.	germacrene-B	1559	0.04	0.01	
53	â-selinene	1485	0.01	0.01	
54.	valencene	1495	0.34	0.15	sweet, woody, citrusy
55.	bicyclogermacrene	1498	0.04	0.03	
56.	á-muurolene	1510	0.07	-	
57.	â-bisabolene	1519	0.07	0.07	
58.	ä-cadinene	1526	0.19	0.09	woody, dry, mild
59.	bergapten	-		0.12	
	Total		1.55	1.43	
	oxides				
60.	caryophillene oxide	1585	0.04	0.03	woody, spicy
61.	cis-limonene oxide	1135	0.03	0.09	citrus like
62.	trans-limonene oxide	1140	0.07	0.09	citrus like
	Total		0.14	0.21	
	ketones				
63.	carvone	1246	0.07	0.09	bitter, spearmint, caraway
64.	nootkatone	1808	0.05	0.03	green, grapefruit
	Total		0.13	0.12	
	acids				
65.	hexadecanoic acid	1890	0.03	0.06	
	Total		0.03	0.06	

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Резиме

Споредбено испитување на етерични масла од сладок и горчлив портокал

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Клучни зборови: *Citrus sinensis, Citrus aurantium,* масло од кора од портокал, GC-MS, испарливи компоненти

Испитуван е составот на испарливата фракција на масло од кората на комерцијални примероци од горчлив и сладок портокл, користејќи ја техниката на гасна хроматографија со масена спектрометрија. Идентификувани се повеќе од 50 компоненти во согласност со нивните масени спектри и релативни ретенциски индекси. Најзастапена компонента е монотерпенот лимонен но не во количините кои можат да бидат очекувани од свежо етерично масло од портокал. Алдехиди, а потоа алкохоли и естери се главните компоненти во оксигенирана фракција на маслото. Во маслото од сладок портокал, алдехидите се доминантни оксигенирани компоненти додека во маслото од горчлив портокал алкохолите и естрите се пронајдени во поголемо количество. Најбитни компоненти за мирисот на етеричното масло од сладок портокал се нонанал, децанал и линалол а за маслото од горчливиот портокал тоа е кетонот карвон во комбинација со други компоненти. Количеството на карвон е индикатор за свежината и староста на маслото додека од количествата и соодносот на α - пинен, β - пинен, сабинен и мирцен може да се процени дали има вештачки промени во природниот состав на етерично масло.

УПАТСТВО ЗА АВТОРИТЕ

Македонскиот фармацевтски билтен е официјално гласило на Македонското фармацевтско друштво. Во списанието се објавуваат оригинални научни трудови, куси соопштенија, ревијални трудови и стручни трудови од сите фармацевтски дисциплини и сродни подрачја на работа кои се од интерес за фармацијата (фармацевтска и медицинска хемија, имунологија со имунохемија, молекуларна биологија, фармацевтски анализи, контрола на лекови, фармацевтска технологија, фармакоинформатика, биофармација, фармакологија, применета ботаника, фармакогнозија, токсикологија, клиничка фармација, храна и исхрана, физичка фармација, органски синтези, социјална фармација, историја на фармација и др.).

Во Македонскиот фармацевтски билтен се објавуваат и други прилози (пораки и соопштенија, извештаи и календари на научни и на стручни манифестации во земјата и во странство, прегледи на книги). Составен дел на Билтенот се и разни рубрики.

Оригиналните научни трудови ги содржат резултатите на сопствени, дотогаш необјавени научни истражувања кои претставуваат заокружена целина, а даваат придонес кон развојот на соодветната научна област. Бројот на страниците (заедно со сликите и со табелите) не треба да биде поголем од 15.

Кусите соопштенија содржат резултати од научни истражувања презентирани во помал обем, чиј број на страници не треба да биде поголем од пет (дозволени се една табела и една слика).

Прегледите се пишуваат на покана од Уредувачкиот одбор, а претставуваат поопширен приказ на истражувањата и сознанијата на повеќе автори во определена научна област, за која компентноста на авторот на прегледот е потврдена преку неговите објавени истражувања.

Стручните трудови известуваат за корисни резултати кои овозможуваат податоците од оригиналните научни истражувања да бидат применети во практичната работа. Стручните трудови можат да се темелат и на обработка на теоретски податоци. Бројот на страниците (заедно со сликите и со табелите) не треба да биде поголем од 10.

Оригиналните научни трудови, кусите соопштенија и прегледите треба да бидат напишани на англиски јазик, додека стручните трудови и сите останати прилози можат да бидат напишани и на македонски јазик.

Прифаќањето на трудовите за печатење во Билтенот ги обврзува авторите истите резултати да не ги објавуваат во друго списание.

Подготовка на ракописот:

Ракописите треба да се достават во три примероци испечатени на компјутерски печатач, еднострано со проред од 1,5 со маргина од 3,0 cm од секоја страна, на хартија со формат А4 на адреса на главниот уредник:

Проф. д-р Светлана Кулеванова
Фармацевтски факултет,
Водњанска 17, 1000 Скопје,
Република Македонија

Страниците и прилозите треба да бидат нумерирани.

При подготвување на ракописот за печатење треба да се води сметка тој да биде максимално концизен, без непотребни повторувања.

При пишување на ракописот задолжителна е употреба на следните единици за: должина (m, cm, mm, μm, nm, Å); маса (kg, g, mg, μg, ng, pg); волумен (l, ml, μl); време (s, min, h, d); температура (°C, K); радијација (Bq, Ci, dpm, Gy, rad): концентрација (M, mol/l, N, mg/ml, %, %(v/v), %(w/v), ppm).

Имињата на супстанциите треба да се во согласност со препораките и правилата дадени од IUPAC или, ако такви не постојат, според практиката на Chemical Abstracts.

Табелите и сликите (фотографии, дијаграми и скици), како и структурните формули треба да бидат дадени секоја на одделен лист (во текстот да е обележано нивното место). Табелите и сликите се нумерираат со арапски бројки (на пр. Табела 1., Сл.1).

Ракописот треба да содржи: наслов, апстракт, клучни зборови, вовед, експериментален дел, резултати и дискусија, заклучок, благодарност (по потреба), литература и резиме.

На **насловната страница** треба да стои насловот на ракописот. Во посебни редови треба да стои полно име и презиме на авторите, назив и адреса на институцијата на авторите. Кога авторите потекнуваат од повеќе различни институции треба да се означи потеклото на секој автор со $^{1, 2, 3}$.... на крајот од неговото име. Со ѕвездичка треба да биде означено името на авторот за коресподенција. Во долниот лев агол од насловната страница се наведува неговата e-mail адреса, телефонскиот број и број на факс.

Апстрактот треба да биде до 250 збора (научни и стручни трудови), односно до 100 збора (куси соопштенија), а треба да ги содржи целта на работата, методологијата и позначајните резултати. Во продолжение треба да бидат застапени три до шест клучни збора распоредени според редоследот на важност.

Во **воведниот дел** треба накусо да се наведат само најважните податоци од поранешните истражувања поврзани со обработуваната проблематика и да се објасни целта и важноста на работата.

Експерименталниот дел треба да биде издвоен и да содржи податоци за употребуваните материјали и опис на применетите методи на начин што ќе овозможи репродуцирање на постапката, но без детално опишување на веќе познатото.

Трудовите што се однесуваат на теоретски истражувања, наместо експериментален дел треба да имаат соодветно поглавје со поднаслови во кое ќе бидат изнесени подробности неопходни за проверка на презентираните резултати.

Резултатите и дискусијата треба да бидат дадени во едно поглавје. Дискусијата треба да содржи анализа на резултатите и на заклучоците што притоа можат да се извлечат.

Заклучокот што произлегува од изложените резултати и дискусија се издвојува во посебно поглавје.

Литературата се наведува во посебно поглавје, при што библиографските единици се нумерираат по оној ред по кој се појавуваат во текстот. Ако во текстот се спомнуваат автори, се наведуваат само презимињата на авторите (ако ги има еден или два), а во случај на повеќе автори се пишува, на пример, *Vetter et al.* (1).

Статии од научни списанија се цитираат на овој начин:

1. P. Premov and D. Dulanov, *Maced. Pharm. Bull.*, **20**, 2-10 (2000).

Кратенките на списанијата се наведуваат според *Chemical Abstracts.*

Книги се цитираат на овој начин:

2. A. Robert, M. Sporn, *Progress in Organic Chemistry,* 3rd ed., Butterworths Scientific Publications, London, 1998, pp 125-154.

Книги со уредник:

3. J. Cook, in *The retinoids,* P. Cornwell, Ed., Academic Press, Orlando, 1996, pp 25-29.

Трудовите напишани на англиски јазик на крајот треба да содржат **резиме** на македонски јазик. Резимето треба да содржи наслов, полно име и презиме на авторот/авторите, назив на институцијата на авторот/авторите, клучни зборови и текстот на апстрактот. Стручните трудови напишани на македонски јазик на крајот треба да содржат резиме напишано на англиски јазик во кое треба да се вклучени истите податоци.

Примените ракописи Уредувачкиот одбор ги испраќа на рецензирање. Рецензираните трудови, заедно со евентуалните забелешки и мислења на Уредувачкиот одбор, се доставуваат до авторите. Тие се должни најдоцна во рок од 30 дена да ги извршат неопходните корекции и финалната верзија на ракописот да ја достават до Уредувачкиот одбор во печатена форма и задолжително и на *PC* форматирана дискета **(Word; Times New Roman, Mac C Times, Macedonian Times** и големина на букви **11).**

Авторите добиваат 20 бесплатни сепарати од секој труд објавен во Билтенот.

INSTRUCTIONS FOR AUTHORS

Macedonian Pharmaceutical Bulletin is an official publication of the Macedonian Pharmaceutical Association. The journal publishes original scientific papers, short communications, reviews and professional papers from all fields of pharmacyand corresponding scientific fields of interest for pharmacy (pharmaceutical and medicinal chemistry, immunology and imunochemistry, molecular biology, pharmaceutical analyses, drug quality control, pharmaceutical technology, pharmacoinformatics, biopharmacy, pharmacology, applied botany, pharmacognosy, toxicology, clinical pharmacy, food and nutrition, physical pharmacy, organical synthesis, social pharmacy, history of pharmacy etc.).

The Macedonian Pharmaceutical Bulletin, also, publishes and other contributions (recommendations and announcements, reports of meetings, important events and dates, book reviews, various rubrics).

Original scientific papers should contain own unpublished results of completed original scientific research. The number of pages (including tables and figures) should not exceed 15.

Short communications also should contain completed but briefly presented results of original scientific research. The number of pages should not exceed 5 (including one table and one figure).

Reviews are written at the invitation of the Editorial Board. They should be surveys of the investigations and knowledge of several authors in a given research area, the competency of the authors of the reviews being assured by their own published results.

Professional papers report on useful practical results which are not original but help the results of the original scientific research to be adopted into practical use. Professional papers might be based on the elaborating of theoretical data. The number of pages should not exceed 10 (including tables and figures).

Original scientific papers, short communications and reviews should be written in

English, while professional papers and all other contributions may be submitted in Macedonian.

Acceptance for publication in the Bulletin obliges the authors not to publish the same results elsewhere.

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Prof. dr. Svetlana Kulevanova
Faculty of Pharmacy
Vodnjanska 17
1000 Skopje
Republic of Macedonia

The pages and appendices should be numbered.

The papers should be written in the shortest possible way and without unnecessary repetition.

Obligatorily, only the follow quantities and units must be used: length (m, cm, mm, μm, nm, Å); weight (kg, g mg, μg, ng, pg); volume (l, ml, μl); time (s, min, h, d); temperature (^oC, K); radiation (Bq, Ci, dpm, Gy, rad); concentration (M, mol/l, N, mg/ml, %, %(v/v), %(w/v), ppm).

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Figures (photographs, diagrams and sketches) and structural formulae should each be given on a separate sheet (the place to which they belong in the text should be indicated). The tables and the figures should be numbered in Arabic numerals (e.g. Table 1, Fig. 1).

Manuscript should contain: title, abstract, key words, introduction, experimental, results and discussion, conclusion, acknowledgment (if desired) references and summary.

Papers should be preceded by a title page comprising: the title, the complete name(s) of the authors, and the author's affiliations (institution and address). When the authors affiliated from various different institutions the origin of the each author should be indicated with 1,2,3... at the end of his name. The correspodence author should be indicated with * and his e-mail address, telephone and fax number to be supplied in the bottom left part of the title page.

Each paper must begin with an Abstract which should not exceed more than 250 (original scientific and professional papers) or 100 (short communications) words. The abstract should include the aim of the research, methods and the most important results. A list of 3 to 6 key words arranged in the order according to their importance should follow the abstract.

In the introduction only the most important previous results related to the problem in hand should be briefly reviewed and the aim and the importance of the research should be stated.

The experimental section should be written as a separate section and should contain a description of the materials used and methods employed in form which makes the results reproducible, but without detailed description of already known methods.

Manuscripts which are related to theoretical studies, instead of experimental, should contain a sub-heading and the theoretical background where the necessary details for verifying the results obtained should be stated.

It is often advantageous to combine Results and Discussion in one section. The discussion should contain an analysis of the results and the conclusions that can be drawn.

Conclusion which is in consequence from the presented results and discussion should be given in the separate section.

The list of references should only include works that are cited in the text and that have been published or accepted for publication. References should be numbered in the order in which they appear in the text and listed in numerical order in the reference list.

The surname of one or two authors may be given in the text, whereas in the case of more than two authors they should be quoted as, for example *Vetter et al.* (1).

Papers from scientific journals should be cited as follows:

1. P. Premov and D. Dulanov, *Maced. Pharm. Bull.* **20**, 2-10 (2000).

The names of journals should be abbreviated as in Chemical Abstracts.

Books should be cited as follows:

2. A. Robert, M. Sporn, *Progress in Organic Chemistry*, 3rd ed., Butterworths Scientific Publications, London, 1998, pp 125-154.

Books with editor:

3. J. Cook, in *The retinoids,* P. Cornwell, Ed., Academic Press, Orlando, 1996, pp 25-29.

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