(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 December 2003 (18.12.2003)

PCT

(10) International Publication Number
WO 03/103683 A1

(51) International Patent Classification[7]: A61K 31/565, A61P 9/10

(21) International Application Number: PCT/NL03/00421

(22) International Filing Date: 11 June 2003 (11.06.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
02077272.9 11 June 2002 (11.06.2002) EP
02077322.2 13 June 2002 (13.06.2002) EP

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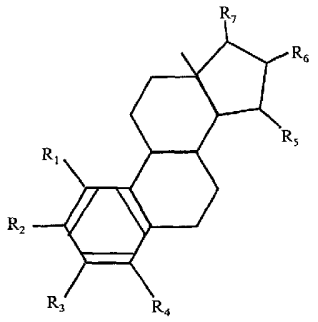
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: USE OF ESTETROL AND ANALOGUES FOR TREATING OR PREVENTING CARDIOVASCULAR PATHOLOGIES, IN PARTICULAR ATHEROSCLEROSIS



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(57) Abstract: The present invention relates to a method of treating or preventing a cardiovascular pathology in a mammal, said method comprising the administration of a therapeutically effective amount of an estrogenic component to said mammal, wherein the estrogenic component is selected from the group consisting of: substances represented by the following formula (I) in which formula R_1, R_2, R_3, R_4 independently are a hydrogen atom, a hydroxyl group or an alkoxy group with 1-S carbon atoms; each of R_5, R_6, R_7 is a hydroxyl group; no more than 3 of R_1, R_2, R_3, R_4 are hydrogen atoms; precursors capable of liberating a substance according to the aforementioned formula when used in the present method; and mixtures of one or more of the aforementioned substances and/or precursors. The method of the invention is particularly suited for treating or preventing hypercholesterolemia, dyslipidemia, atherosclerosis, arteriosclerosis, xanthomatosis myocardial infarction, stroke, multiple silent stroke, unstable angina, loss of cognitive function, vascular dementia and Alzheimer disease.

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USE OF ESTETROL AND ANALOGUES FOR TREATING OR PREVENTING CARDIOVASCULAR PATHOLOGIES, IN PARTICULAR ATHEROSCLEROSIS

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a method for treating or preventing cardiovascular pathologies in a mammal by administering an effective amount of an estrogenic component to said mammal. The method is particularly suited for treating or preventing hypercholesterolemia, dyslipidemia, atherosclerosis, arteriosclerosis, xanthomatosis myocardial infarction, stroke, multiple silent stroke, unstable angina, loss of cognitive function, vascular dementia and Alzheimer disease.

BACKGROUND OF THE INVENTION

Hypercholesterolemia is a condition with elevated levels of circulating total cholesterol, low density lipoprotein cholesterol and very low density lipoprotein cholesterol (hereafter referred to as LDL-C and VLDL-C) as per the ATP guidelines (see JAMA 2000). In particular, high levels of LDL-C and VLDL-C are positively associated with coronary arteriosclerosis while high levels of high density lipoproteins cholesterol (HDL-C) are negative risk factors.

The term "lipoprotein" refers to a group of proteins, found in the serum, plasma and lymph, that are important for lipid transport (e.g. VLDL, LDL and HDL). Cholesterol synthesised *de novo* is transported from the liver and intestine to peripheral tissues in the form of lipoproteins. The chemical composition of each lipoprotein differs in that the HDL has a higher proportion of protein versus lipid, whereas the VLDL has a lower proportion of protein versus lipid.

"Apolipoprotein B" or "Apo B" is a protein component of the LDL cholesterol (LDL-C) transport proteins. Most of the apolipoprotein B is secreted into the circulatory system as VLDL. "Apolipoprotein A1" ("ApoA1") and "apolipoprotein A2" ("ApoA2") are protein components of the HDL cholesterol (HDL-C) transport proteins. Consequently, high levels of apolipoprotein B are positively associated with coronary arteriosclerosis while high levels of apolipoprotein A1 and A2 are negative risk factors. "Lipoprotein (a)"or "(Lp(a)" is commonly used to refer LDL-C plus apolipoprotein (a).

The role of LDL-C oxidation is gaining much attention in the literature. It is well documented that LDL-C becomes oxidatively stressed under pathological conditions and is no longer recognised by the LDL-C receptors. The oxidised LDL-C is taken up by macrophages within the subendothelial space, leading to the formation of fatty streaks which are the basis of most advanced lesions.

Hypercholesterolemia is implicated as a high risk factor of cardiovascular pathologies, including arteriosclerosis, atherosclerosis and xanthomatosis in humans. Hypercholesterolemia is influenced by diet, heredity, environment, life style, diseases and stress, leading to heart attacks and strokes at an early age.

Dyslipidemia is a condition where the blood lipid parameters are abnormal, e.g. elevated levels of LDL-C and VLDL-C, elevated level of Lp(a), low HDL-C or combinations thereof.. The lipids fractions in the circulating blood, mainly cholesterol, triglycerides and phospholipids, are carried within lipoproteins, chylomicronen, LDL-C, VLDL-C and HDL-C. As per the American Heart Association guidelines, the safe levels are represented below. Active treatment by diet modifications and drugs are necessary to reduce the risk of fatality when the levels go abnormal. Of particular importance is a low LDL-C level, e.g. of less than 100 mg/dL, in high risk individuals for myocardial infarction and individuals in secondary prevention. Ideally, in such individuals, HDL-C exceeds 35 mg/dL, triglycerides are below 150 mg/dL and Lp(a) below 40 mg/dL.

Dyslipidemia results from diet, heredity, lifestyle (smoking), environment, familial diseases, or stress. The condition may be inherited or may be secondary to another disorder, such as Systemic Lupus Erythematosus (SLE), Hypothyroidism, Nephrotic Syndrome, Cushing's Syndrome, Diabetes Mellitus, obesity, alcoholism or Corticosteroid Therapy. Dyslipidemia predisposes a subject to coronary heart disease, cerebrovascular disease, peripheral artery disease and obesity. Dyslipidemia is one of the high risk factors useful in the early diagnosis of these life threatening diseases. To some extent, dyslipidemia can be corrected by diet modifications and treatment with drugs.

Arteriosclerosis is the general term for localised arterial lesions that manifest themselves as reconstruction, hardening, and/or hypofunction on the arterial wall. Among other such phenomena, atherosclerosis is clinically particularly important. Atherosclerosis may occur in coronary arteries, cerebral arteries, renal arteries and arteries of the limbs, leading to a narrowing of the internal arterial diameter and formation of thrombosis, which in turn may induce myocardial infarction, cerebral restraint, renal restraint or necrosis of the limbs.

Atherosclerosis is the leading cause of death in western industrialised countries. Atherosclerotic heart disease involving the coronary arteries is the most common single cause of death. Atherosclerotic interference with blood supply to the brain (causing stroke) is the third most common cause of death after cancer. Atherosclerosis also causes a great deal of serious illness by reducing the blood flow in other major arteries, such as those to the kidneys, the legs and the intestines.

Atherosclerosis is a cardiovascular condition occurring as a result of narrowing down of the arterial walls. The narrowing is due to the formation of plaques (raised patches) or streaks in the inner lining of the arteries. These plaques consist of foam cells of low-density lipoproteins, oxidised-LDL-C, decaying muscle cells, fibrous tissue, clumps of blood platelets, cholesterol, and sometimes calcium. They tend to form in regions of turbulent blood flow and are found most often in people with high concentrations of cholesterol in the bloodstream. The number and thickness of plaques increase with age, causing loss of the smooth lining of the blood vessels and encouraging the formation of thrombi (blood clots). Sometimes fragments of thrombi break off and form emboli, which travel through the bloodstream and block smaller vessels.

The major causes of atherosclerosis are hypercholesterolemia and dyslipidemia. The risk of developing atherosclerosis is directly related to plasma levels of LDL-C and inversely related to HDL-C levels. Over 20 years ago, the pivotal role of the LDL-C receptor in LDL-C metabolism was elucidated by Goldstein, et al., in the Metabolic and Molecular Bases of Inherited Disease, Scriver, et al. (McGraw-Hill, NY 1995), pp. 1981-2030. In contrast, the cellular mechanisms responsible for HDL-C metabolism are still not well defined. It is generally accepted that HDL-C is involved in the transport of cholesterol from extrahepatic tissues to the liver, a process known as reverse cholesterol transport, as described by Pieters, et al., Biochim. Biophys. Acta 1225, 125 (1994), and mediates the transport of cholesteryl ester to steroidogenic tissues for hormone synthesis, as described by Andersen and Dietschy, J. Biol. Chem. 256, 7362 (1981). The mechanism by which HDL-C is delivered to target cells differs from that of LDL-C. The receptor-mediated metabolism of LDL-C has been thoroughly described and involves cellular uptake and degradation of the entire particle. In contrast, the receptor-mediated HDL-C metabolism has not been understood as well. Unlike LDL-C, the protein components of HDL-C are not degraded in the process of transporting cholesterol to cells.

Xanthomatosis is a clinical phenotype evidenced by a yellowish swelling or plaques in the skin resulting from deposits of fat. The presence of xanthomas are usually accompanied by raised blood LDL-C levels.

In a review article by Subbiah ("Estrogen replacement therapy and cardioprotection: mechanisms and controversies", Braz J Med Biol Res (2002), Mar;35(3):271-276) it is reported that epidemiological and case-controlled studies suggest that estrogen replacement therapy might be beneficial in terms of primary prevention of coronary heart disease. It is observed that this beneficial effect of estrogens was initially considered to be due to the reduction of low density lipoproteins (LDL-C) and to increases in high density lipoproteins (HDL-C), but that recent studies have shown that estrogens protect against oxidative stress and decrease LDL-C oxidation. The estrogen most commonly used in estrogen replacement therapy is 17β-estradiol.

US-B 6,207,659 describes a method of reducing arterial accumulation and degradation of low density lipoproteins (LDL-C) in a mammal, comprising administering a therapeutically effective amount of 17α-dihydroequilenin or a mammalian metabolic conjugate thereof to a mammal in need of such treatment. 17α-dihydroequilenin is constituent of conjugated equine estrogens. In the US patent is stated that 17α-dihydroequilenin can reduce and prevent atherogenesis by 1) preventing endothelium-dependent vasoconstriction in both males and menopausal females; 2) increasing apolipoprotein A-1; 3) improving insulin sensitivity (while decreasing serum insulin concentrations without reducing glucose concentrations); 4) reducing LDL-C accumulation; and 5) reducing arterial peroxidation.

17β-estradiol which is widely used in hormone replacement therapy is known to exhibit serious pharmacokinetic deficits. The oral bio-availability of this estrogen is very low and varies greatly from person to person, meaning that general dosage recommendations cannot be given. Fast elimination of 17β-estradiol from the blood is another related problem; its half-life is around 1 hour. As a result, between separate (daily) administration events, blood serum levels of this estrogen tends to fluctuate considerably. Thus, shortly after administration the serum concentration is usually several times higher than the optimum concentration. In addition, if the next administration event is delayed, serum concentrations will quickly decrease to a level where the estrogen is no longer physiologically active.

Seeger et al., "The inhibitory effect of endogenous estrogen metabolites on copper-mediated odixation of LDL", Int J Clin Pharm Therap (1998); 36; 383-395, describe the results of an *in vitro* study in which the anti-oxidant effect of 17β-estradiol, its main A- and D-ring metabolites and of vitamin E were compared. It is concluded that the A-ring

metabolites of estradiol may be involved in the physiologic inhibition of LDL-oxidation. All the A-ring metabolites inhibited the onset of LDL oxidation to a significantly greater extent than the parent substance estradiol. The D-ring metabolites and vitamin E were similar to estradiol in their potency. Estetrol was one of the D-ring metabolites that was investigated.

Mueck et al., "Angiogenetic and Anti-Angiogenetic Effects of Estradiol and its Metabolites", J Clin Basic Cardiol (2001); 4; 153-155, report the results of an *in vitro* study in which the angiogenetic and anti-angiogenetic properties of A-ring and D-ring metabolites of 17β-estradiol are compared with that of their parent substance. According to the authors angiogenesis may be important for the development of atherosclerotic plaques and long-term anti-angiogenetic therapy may present an effective new-anti-atherosclerotic approach. It was found that 17β-estradiol and the hydroxylated A-ring metabolites showed a biphasic reaction on the proliferation of vascular endothelial cells. At low concentrations it stimulated and at high concentrations it inhibited cell growth. For the D-ring metabolites, including estetrol, no marked changes were observed.

In addition to pharmacokinetic problems, 17β-estradiol as well as other commonly used estrogens show pharmacodynamic deficits. After resorption from the intestinal lumen, orally applied active ingredients enter the organism via the liver. This fact is of specific importance for estrogenic agents as the liver is a target organ for estrogens; oral intake of estrogens results in strong estrogenic effects in the liver. The secretion activity that is controlled by estrogens in the human liver includes increased synthesis of transport proteins CBG, SHBG, TBG, several factors that are important for the physiology of blood clotting, and lipoproteins. If biogenic estrogens (i.e. estrogens occurring naturally in the human body) are introduced to the female organism while avoiding passage through the liver (e.g. by transdermal application), the liver functions mentioned remain largely unchanged. Therapeutically equivalent doses of commonly known biogenic estrogens, when applied orally, result in clear responses of hepatic parameters, such as increase of SHBG, CBG, and angiotensinogen.

Consequently there is a need for estrogenic substances that:

(a) are effective in a method of treating or preventing cardiovascular pathologies, especially in individuals at high risk or secondary prevention (post myocardial infarction. percutaneous transluminal coronary angioplast (PTCA) or coronary artery bypass graft (CABG)), unstable angina, multiple silent stroke, loss of cognitive function, vascular dementia, Alzheimer disease and

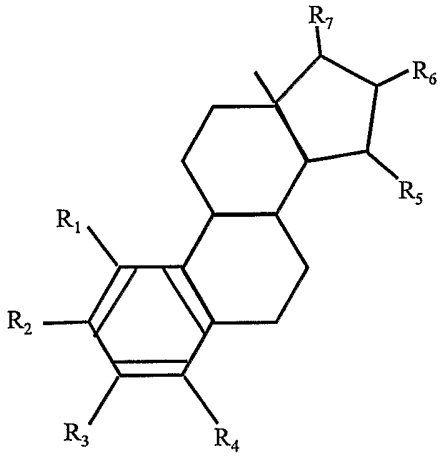
(b) have a significantly longer half-life than estrogens that have been proposed for such treatments and/or

(c) are orally administerable without causing significant hepatic effects and/or

(d) produce less undesirable side-effects than other estrogens that have been used or recommended for the treatment of cardiovascular pathologies..

SUMMARY OF THE INVENTION

The inventors have surprisingly found that these requirements are met by estrogenic substances that are represented by the following formula



in which formula R_1, R_2, R_3, R_4 independently are a hydrogen atom, a hydroxyl group or an alkoxy group with 1-5 carbon atoms; each of R_5, R_6, R_7 is a hydroxyl group; and no more than 3 of R_1, R_2, R_3, R_4 are hydrogen atoms.

A known representative of this group of estrogenic substances is 1,3,5 (10)-estratrien-3, 15α,16α,17β-tetrol, also known by the names of estetrol, oestetrol and 15α-hydroxyestriol. Estetrol is an estrogen that is produced by the fetal liver during human pregnancy. Unconjugated estetrol levels in maternal plasma peak at about 1.2 ng/ml at term pregnancy and are about 12 times higher in fetal than in maternal plasma (Tulchinsky et al., 1975. J. Clin. Endocrinol. Metab., 40, 560-567).

The inventors have found that the present estrogenic substances are capable of influencing blood lipids factors in a way that reduces the risk of cardiovascular pathologies, especially when taken orally. Blood lipid factors that are considered to be risk factors of

cardiovascular pathologies and that can be reduced by administration of the present estrogenic substances include serum levels of total cholesterol, LDL-C, apolipoprotein B and triglycerides. In addition, the present estrogenic substances may exert a favourable effect by increasing the serum concentration of HDL-C and by preventing LDL-C-oxidation.

In 1970, Fishman et al., "Fate of 15α-hydroxyestriol-^{3}H in Adult Man", J Clin Endocrinol Metab (1970) 31, 436-438, reported the results of a study wherein tritium labeled 15α-hydroxyestriol (estetrol) was administered intravenously to two adult women. It was found that the estetrol was rapidly and completely excreted in urine as the glucosiduronate and that virtually no metabolism except for conjugation took place.

Between 1975 and 1985 several researchers have investigated the pharmaceutical properties of estetrol and reported on its estrogenic potency and uterotrophic activity. The most relevant publications that were issued during this period are mentioned below:

- Levine et al., 1984. Uterine vascular effects of estetrol in nonpregnant ewes. Am. J. Obstet. Gynecol., 148:73, 735-738: "When intravenously administered in nonpregnant ewes, estetrol is 15 to 30 times less potent than estriol and 17β-estradiol in uterine vasodilation".
- Jozan et al., 1981. Different effects of oestradiol, oestriol, oestetrol and of oestrone on human breast cancer cells (MCF-7) in long term tissue culture. Acta Endocrinologica, 98, 73-80: "Estetrol agonistic potency is 2% of the magnitude observed for 17β-estradiol in in vitro cell proliferation".
- Holinka et al., 1980. Comparison of effects of estetrol and tamoxifen with those of estriol and estradiol on the immature rat uterus. Biol. Reprod. 22, 913-926: "Subcutaneously administered estetrol has very weak uterotrophic activity and is considerable less potent than 17β-estradiol and estriol".
- Holinka et al., 1979. In vivo effects of estetrol on the immature rat uterus. Biol. Reprod. 20, 242-246: "Subcutaneously administered estetrol has very weak uterotrophic activity and is considerable less potent than 17β-estradiol and estriol".
- Tseng et al., 1978. Heterogeneity of saturable estradiol binding sites in nuclei of human endometrium. Estetrol studies. J. Steroid Biochem. 9, 1145-1148: "Relative binding of estetrol to estrogen receptors in the human endometrium is 1.5 % of 17β-estradiol".
- Martucci et al., 1977. Direction of estradiol metabolism as a control of its hormonal action-uterotrophic activity of estradiol metabolites. Endocrin. 101, 1709-1715:

"Continuous administration of estetrol from a subcutaneous depot shows very weak uterotrophic activity and is considerably less potent than 17β-estradiol and estriol".

- Tseng et al., 1976. Competition of estetrol and ethynylestradiol with estradiol for nuclear binding in human endometrium. J. Steroid Biochem. 7, 817-822: "The relative binding constant of estetrol binding to the estrogen receptor in the human endometrium is 6.25% compared to 17β-estradiol (100%)".
- Martucci et al., 1976. Uterine estrogen receptor binding of catecholestrogens and of estetrol (1,3,5(10)-estratriene-3,15alpha,16alpha, 17beta-tetrol). Steroids, 27, 325-333: "Relative binding affinity of estetrol to rat uterine cytosol estrogen receptor is 0.5% of 17β-estradiol (100%). Furthermore, the relative binding affinity of estetrol to rat uterine nuclear estrogen receptor is 0.3% of 17β-estradiol (100%)".

All of the above publications have in common that the authors have investigated the estrogenic potency of estetrol. Without exception they all conclude that estetrol is a weak estrogen. In some of the cited articles the estrogenic potency of estetrol has been found to be much lower than that of the widely used and relatively weak estrogen 17β-estradiol. With these findings in mind, it is not surprising that the interest in estetrol has dwindled since the early eighties and that no publications on the properties of estetrol have been issued since.

The inventors have surprisingly found that, despite its low potency, estetrol as well as related estrogenic substances may advantageously be used in a method of treating or preventing cardiovascular pathologies. Although the inventors do not wish to be bound by theory, it is believed that the unexpected efficacy of the present estetrol-like substances results from the combination of the unexpected favourable pharmacodynamic and pharmacokinetic properties of these substances.

As regards the pharmacokinetic properties of the present estrogenic substances the inventors have discovered that their *in vivo* half-life is considerably longer than the half-life of estrogens that have been proposed for treating cardiovascular pathologies, notably 17β-estradiol and 17α-dihydroequilenin. Thus, even though estetrol and estetrol-like substances have relatively low estrogenic potency, they may effectively be employed in a method of treating or preventing cardiovascular pathologies because their low potency is compensated for by a relatively high metabolic stability as demonstrated by a long half-life.

In addition, it is believed that the unexpected efficacy of the present estetrol-like substances may be explained by the relatively high affinity for the estrogen receptor α (ERα) as compared to the estrogen receptor β (ERβ). The latter characteristic is an unique feature of

the estrogenic substances employed in the present method. The relatively high affinity of the present estrogenic substances for the ERα receptor, or conversely the relatively low affinity for the ERβ receptor, is believed to be somehow associated with the high efficacy of the present substances for treating or preventing cardiovascular pathologies.

A recent publication strongly suggests that the ERα gene expression plays a key role in lipid metabolism of mammals. Ohlsson et al., "Obesity and disturbed lipoprotein profile in estrogen receptor-α-deficient male mice", Biochem Biophys Res Commun (2000); 278, 640-645 reports the results of studies into the lipid metabolism of ERα knockout (ERKO), ERβ knockout (BERKO), and ERα/β double knockout (DERKO) mice. It was found that adult male ERKO and DERKO mice that were deficient for the ERα, unlike BERKO mice that are deficient for the ERβ, gene, demonstrated a clear increase in total body fat and enhanced serum leptin levels. In both the ERKO and DERKO mice serum cholesterol was increased and a qualitative change in the lipoprotein profile, including smaller LDL-C particles, was observed. In conclusion, the presence, and possibly signalling through ERα appears to be a prerequisite for proper lipid metabolism and a favourable lipid profile.

Thus, although the mechanisms by which the present estrogenic substances exert their favourable effect are not fully understood, it is evident that these substances differ from other biogenic estrogens in 2 important aspects. Firstly the present estrogenic substances exhibit a surprisingly long *in vivo* half-life. Secondly the ratio between the affinity of these substances for the ERα and the ERβ receptor is much higher and more favourable than that of other known (biogenic) estrogens.

Another advantageous property of the present estrogenic substances resides in the fact that sex hormone-binding globulin (SHBG) hardly binds these estrogenic substances, meaning that, in contrast to most known estrogens, serum levels are representative for bio-activity and independent of SHBG levels.

Yet another important benefit of the present estrogenic substances is derived from their relative insensitivity to interactions with other drugs (drug-drug interactions). It is well known that certain drugs may decrease the effectiveness of estrogens and other drugs may enhance their activity, resulting in possible increased side-effects. Similarly estrogens may interfere with the metabolism of other drugs. In general, the effect of other drugs on estrogens is due to interference with the absorption, metabolism or excretion of these estrogens, whereas the effect of estrogens on other drugs is due to competition for metabolic pathways.

The clinically most significant group of estrogen-drug interactions occurs with drugs that may induce hepatic microsomal enzymes which may decrease estrogen serum levels

below therapeutic level (for example, anticonvulsant agents; phenytoin, primidone, barbiturates, carbamazepine, ethosuximide, and methosuximide; antituberculous drugs such as rifampin; antifungal drugs such as griseofulvin). The present estrogenic substances are less dependent on up- and downregulation of microsomal liver enzymes (e.g. P450's) and also are less sensitive to competition with other P450 substrates. Similarly, they do not interfere significantly in the metabolism of other drugs.

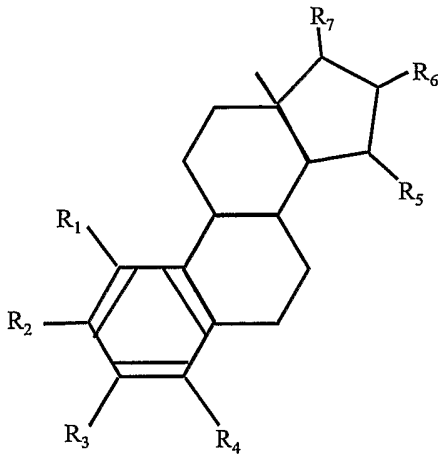
The conjugates of most estrogens, as formed in the liver, are excreted in the bile and may be broken down by gut bacteria in the colon to liberate the active hormone which can then be reabsorbed (enterohepatic recirculation). There are clinical reports that support the view that enterohepatic recirculation of estrogens decreases in women taking antibiotics such as ampicillin, tetracycline, etc. Conjugated forms of the present estrogenic substances are hardly excreted in the bile, meaning that they are substantially insensitive to drugs that do influence the enterohepatic recirculation of other estrogens.

The above observations serve to explain why the estrogenic substances of the invention hardly suffer from drug-drug interactions and thus produce a very consistent, i.e. predictable, impact.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of treating or preventing a cardiovascular pathology in a mammal, said method comprising the administration of a therapeutically effective amount of an estrogenic component to said mammal, wherein the estrogenic component is selected from the group consisting of:

substances represented by the following formula



in which formula R_1, R_2, R_3, R_4 independently are a hydrogen atom, a hydroxyl group or an alkoxy group with 1-5 carbon atoms; each of R_5, R_6, R_7 is a hydroxyl group; no more than 3 of R_1, R_2, R_3, R_4 are hydrogen atoms; precursors capable of liberating a substance according to the aforementioned formula when used in the present method; and mixtures of one or more of the aforementioned substances and/or precursors.

As used herein the term "cardiovascular pathology" is a disease or disorder of the blood vessels of the circulation system caused by or associated with abnormally high concentrations of lipids in the vessels.

The present estrogen substances are distinct from both the biogenic and synthetic estrogens that are commonly applied in pharmaceutical formulations in that they contain at least 4 hydroxyl groups. The present substances are particularly special in that the 5 membered ring in the steroid skeleton comprises 3 hydroxyl substituents rather than 0-2. Examples of commercially available estrogens that contain at least 4-hydroxyl groups and their precursors are:

1, 3, 5(10)-estratrien-2, 3, 15α, 16α, 17β- pentol 2-methyl ether

1, 3, 5(10)-estratrien-2, 3, 15β, 16α, 17β- pentol 2-methyl ether

1, 3, 5(10)-estratrien-2, 3, 16α, 17β- tetrol

1, 3, 5(10)-estratrien-3, 4, 16α, 17β- tetrol 4-methyl ether

1, 3, 5(10)-estratrien-3, 15α, 16α, 17β- tetrol

1, 3, 5(10)-estratrien-3, 15α, 16α, 17β- tetrol tetra acetate

1, 3, 5(10)-estratrien-3, 15β, 16β, 17β- tetrol tetra acetate

Preferably, the estrogenic component applied as the active component in the present composition is a so called biogenic estrogen, i.e. an estrogen that occurs naturally in the

human body, a precursor of a biogenic estrogen or a mixture thereof. Because biogenic estrogens are naturally present in the fetal and female body, side-effects are not expected to occur, particularly not if the serum levels resulting from the exogenous administration of such estrogens do not substantially exceed naturally occurring concentrations.

In a preferred embodiment of the present invention the estrogenic substance contains 4 hydroxyl groups. Also, in the aforementioned formula, R_1 preferably represents a hydrogen atom. In said formula preferably at least 2, more preferably at least 3 of the groups R_1, R_2, R_3 and R_4 represent a hydrogen atom.

The estrogenic substances according to the formula encompass various enantiomers since the carbon atoms that carry hydroxyl-substituents R_5, R_6 and R_7 are chirally active. In one preferred embodiment, the present estrogenic substance is 15α-hydroxy substituted. In another preferred embodiment the substance is 16α-hydroxy substituted. In yet another preferred embodiment, the substances is 17β-hydroxy substituted. Most preferably the estrogenic substances are 15α,16α,17β-trihydroxy substituted.

In a preferred embodiment of the present invention R_3 represents a hydroxyl group or an alkoxy group. In another preferred embodiment the groups R_1, R_2 and R_4 represent hydrogen atoms, in which case, if R_3, R_5, R_6 and R_7 are hydroxyl groups, the substance is 1,3,5 (10)-estratrien-3, 15,16,17-tetrol. A preferred isomer of the latter substance is 1,3,5 (10)-estratrien-3, 15α,16α,17β-tetrol (estetrol).

The invention also encompasses the use of precursors of the estrogen substances that constitute the active component in the present method. These precursors are capable of liberating the aforementioned estrogen substances when used in the present method, e.g. as a result of metabolic conversion. These precursors are preferably selected from the group of derivatives of the present estrogen substances, wherein the hydrogen atom of at least one of the hydroxyl groups has been substituted by an acyl radical of a hydrocarbon carboxylic, sulfonic acid or sulfamic acid of 1-25 carbon atoms; tetrahydrofuranyl; tetrahydropyranyl; or a straight or branched chain glycosydic residue containing 1-20 glycosidic units per residue. Typical examples of precursors which can suitably be used in accordance with the invention are esters that can be obtained by reacting the hydroxyl groups of the estrogen substances with substances that contain one or more carboxy (M^+ $^-$OOC-) groups, wherein M^+ represents a hydrogen or (akali)metal cation. Hence, in a particularly preferred embodiment, the precursors are derivatives of the estrogen substances, wherein the hydrogen atom of at least one of the hydroxyl groups in said formula has been substituted by -CO-R, wherein R is a hydrocarbon radical comprising from 1-25 carbon atoms. Preferably R is hydrogen, or an

alkyl, alkenyl or aryl radical comprising from 1-20 carbon atoms.

The present method is particularly effective when the administration is continued for a prolonged period of time. Usually, the method comprises the uninterrupted administration of the estrogenic component during a period of at least 5 days. Preferably the uninterrupted administration is continued for at least 30 days, more preferably for at least 90 days. The present method may suitably employ enteral or parenteral administration of the estrogenic component. The term "parenteral administration" as used in here encompasses transdermal, intravenous, intranasal, intravaginal, pulmonary, buccal, subcutaneous, intramuscular and intra-uterine administration. The term "enteral administration" includes oral as well as rectal administration.

Preferably the mode of administration is selected from the group consisting of oral, transdermal, intravenous, intranasal, intravaginal, pulmonary, rectal, buccal, subcutaneous, intramuscular or intra-uterine administration. More preferably the mode of administration is selected from the group consisting of oral, transdermal, intravenous, subcutaneous, intranasal, pulmonary and vaginal administration. In a particularly preferred embodiment the present method employs oral, transdermal, intranasal or subcutaneous administration. Even more preferably the present method employs oral or transdermal administration.

Oral, intravenous, subcutaneous, intramuscular, intranasal, rectal, buccal and pulmonary administration are ideally suited for (at least) once daily administration. Transdermal administration is advantageously applied at frequencies between once a day and once a month. Intravaginal and intra-uterine administrations are advantageously operated at administration frequencies between once weekly and once monthly. Subcutaneous and intramuscular administration may also suitably be done in the form of depot injections at intervals of 1 week to 6 months, preferably at intervals of 4 weeks to 3 months.

For reasons of convenience, the present method preferably utilises administration intervals of 1 day, 1 week or 1 month. Regimens that employ once daily oral, subcutaneous, intravenous or intranasal administration, once weekly transdermal or once monthly intravaginal or subcutaneous administration are particularly preferred.

Irrespective of the mode of administration, the estrogenic component is preferably administered in an amount effective to achieve a blood serum concentration of at least 1 nanogram per litre, more preferably of at least 10 nanogram per litre, most preferably at least 100 nanogram per litre. Generally the resulting blood serum concentration of the estrogenic component will not exceed 100 μg per litre, preferably it will not exceed 50 μg per litre, more preferably it will not exceed 25 μg per litre.

In accordance with the present method the estrogenic component is usually administered in an amount of less than 1 mg per kg of bodyweight per day, preferably of less than 0.4 mg per kg of bodyweight per day. In order to achieve a significant impact from the administration of the estrogenic component, it is advisable to administer in an amount of at least 1 μg per kg of bodyweight per day. Preferably, the administered amount is at least 5 μg per kg of bodyweight per day.

Oral administration of the active component is preferably done in an amount of less than 400 μg per kg of bodyweight per day, preferably of less than 200 μg per kg of bodyweight per day. In order to achieve a significant impact from the administration of the active component, it is advisable to orally administer in an amount of at least 2 μg per kg of bodyweight per day. Preferably, the orally administered amount is at least 5 μg per kg of bodyweight per day. In the present method, particularly when used in humans, the estrogenic component is usually administered in an average dosage of at least 0.05 mg per day, preferably of at least 0.1 mg per day. The maximum dosage is normally kept below 40 mg per day, preferably below 20 mg per day.

The present method of treatment preferably comprises administering to a person in need of such a therapy an effective amount of the estrogenic component. The amounts needed to be effective will differ from individual to individual and are determined by factors such as the individual's level of estrogen deficiency, body weight, route of administration and the efficacy of the particular estrogenic substance used.

In the present method, particularly when used in humans, the estrogenic component is usually orally administered in an average dosage of between 0.01 and 20 mg per day, preferably of between 0.05 and 10 mg per day. Similarly, the parenteral dosage preferably is at least 0.05, preferably at least 0.1 mg per day. The average maximum parenteral dosage is normally kept below 40 mg per day, preferably below 20 mg per day.

In a particularly preferred embodiment of the invention the method employs oral administration of the active estrogenic component. The term oral administration as used in here also encompasses oral gavage administration. The inventors have surprisingly found that, despite its low potency, estetrol and related estrogenic substances may advantageously be administered orally. Although the inventors do not wish to be bound by theory, it is believed that the unexpected efficacy of orally administered estetrol-like substances results from the combination of special pharmacokinetic (ADME) and pharmacodynamic properties of these substances.

The inventors have discovered that the oral bioavailability of estetrol-like substances is surprisingly high and that their *in vivo* half-life is considerably longer than that of commonly used biogenic estrogens. Thus, even though estetrol and estetrol-like substances have relatively low estrogenic potency, they may effectively be administered orally because the oral dosages required to achieve the desired effect are similar to those already used for e.g. 17β-estradiol.

Another important advantage of oral administration of estetrol and estetrol-like substances resides in the fact that the hepatic effects of these substances are deemed to be minimal since they are hardly metabolised during the so called "first pass". The first-pass effect of drugs given orally refers to the process of drug degradation by the liver during a drug's transition from initial ingestion to circulation in the blood stream. After resorption from the intestinal lumen, orally applied active ingredients enter the organism via the liver. This fact is of specific importance for estrogenic agents as the liver is a target organ for estrogens; oral intake of estrogens results in strong estrogenic effects in the liver. Therapeutically equivalent doses of commonly used biogenic estrogens, when applied orally, result in clear responses of hepatic parameters, such as increase of SHBG, CBG and angiotensinogen.. These hepatic effects of estrogens are also observed when equine estrogen formulations (so-called conjugated estrogens) are used.

The present method may suitably be used in the (prophylactic) treatment of a wide variety of cardiovascular pathologies. The method is suited for treating or preventing cardiovascular pathologies selected from the group consisting of hypercholesterolemia, dyslipidemia, atherosclerosis, arteriosclerosis, xanthomatosis, myocardial infarction, stroke, multiple silent stroke, unstable angina, loss of cognitive function, vascular dementia and Alzheimer disease. Cardiovascular disorders that may be treated particularly effectively include hypercholesterolemia, dyslipidemia, atherosclerosis, arteriosclerosis, myocardial infarction and stroke. Best results are obtained in the treatment of hypercholesterolemia and atherosclerosis.

As used herein the term "arteriosclerosis" is a degeneration of the walls of the arteries due to the formation of foam cells and aortic streaks which narrow the arteries. This limits blood circulation and predisposes an individual to thrombosis. As used herein the term "atherosclerosis" is a disease of the arteries in which fatty plaques develop on the inner walls, with eventual obstruction of blood flow.

The present method is particularly suitable for treating patients whose blood lipid parameters indicate that they are at increased risk of a cardiovascular pathology such as a

myocardial infarction or a stroke. Consequently, the present method is suitably used to treat a mammal, especially a human patient, whose total blood cholesterol level exceeds 240 mg/dL and/or blood LDL-C level exceeds 100 mg/dL and/or blood HDL-C is below 35 mg/dL and/or blood triglycerides level exceeds 150 mg/dL. The present method is particularly suitable for treating human patients whose blood LDL-C level exceeds 100 mg/dl, especially those patients whose blood LDL-C level exceeds 130 mg/dL and in particular those patients whose blood LDL-C level exceeds 160 mg/dL. The dosages employed in the present method may vary considerably in dependency of the individuals physiology and the seriousness of his/her affliction. Preferably the method comprises administering the estrogenic component in an amount which is effective in reducing the serum level of low density lipoprotein cholesterol(LDL-C). Similarly, the administered amount of the estrogenic component is preferably effective to reduce the serum level of apolipoprotein B. As used herein the term "apolipoprotein B" refers to the protein component of the LDL-C transport proteins. Cholesterol synthesised *de novo* is transported from the liver and intestine to peripheral tissues in the form of lipoproteins. Most of the apolipoprotein B is secreted into the circulatory system as VLDL-C. In another preferred embodiment, the estrogenic component is administered in an amount effective to increase the serum level of high density lipoprotein cholesterol (HDL-C) . Generally the combined effect of administration of the present estrogenic component on serum LDL-C and HDL-C levels is such that a significant increase in the HDL-C:LDL-C ratio is observed.

In yet another preferred embodiment the estrogenic component is administered in an amount effective to prevent *in vivo* oxidation of low density lipoprotein. Oxidised LDL-C cannot be recognised by LDL-C receptors and is taken up by macrophages within the subendothelial space, leading to the formation of fatty streaks that are the basis of most advanced lesions.

In another preferred embodiment of the invention the present method comprises the co-administration of a progestogen, particularly if the method is employed in the treatment of female mammals. The administration of estrogens has been associated with endometrial proliferation in women and it is now widely accepted that "unopposed" estrogen administration during a prolonged period of time (estrogen therapy) substantially increases the risk of endometrial cancer (Cushing et al., 1998. Obstet. Gynecol.91, 35-39; Tavani et al., 1999. Drugs Aging, 14, 347-357). There is also evidence of a significant increase in breast cancer with long-term (10-15 years) use of estrogen therapy (Tavani et al., 1999. Drugs Aging, 14, 347-357; Pike et al., 2000. Steroids, 65, 659-664).

In order to counteract the negative effects of prolonged unopposed estrogen therapy, adjunctive progestogen treatment is nowadays commonly applied in hormone replacement therapy in peri- and postmenopausal women. Regular progestogen administration is believed to inhibit the continual estrogen stimulation of the endometrium through an anti-proliferative effect and appears to reduce the incidence of endometrial carcinoma in post-menopausal women receiving estrogen replacement therapy (Beral et al., 1999. J. Epidemiol. Biostat., 4, 191-210). In order to counteract any potential negative effects of unopposed estrogen administration in the present method, particularly in case of prolonged continuous administration, it is preferred to co-administer a progestogenic component to inhibit estrogen stimulation of the endometrium or to administer a progestogenic component at least during a period of ten days at least every three months.

The present estrogenic component can be suitably administered in any form of pharmaceutical formulation known in the art. The pharmaceutical formulation can be a solid or semi-solid dosage form such as tablets, capsules, cachets, pellets, pills, powders and granules, as well as fluid dosage forms such as solutions, emulsions, suspensions, ointments, pastes, creams, gels, jellies and foams.

Examples of oral dosage units that may be used in the present method include solid or semi-solid dosage forms such as tablets, capsules, cachets, pellets, pills, powders and granules. The term "solid or semi-solid dosage form" also encompasses capsules that contain a liquid, e.g. an oil, in which the present estrogenic component is dissolved or dispersed. Tablets and equivalent solid and semi-solid dosage forms can suitably contain materials such as binders (e.g. hydroxypropylmethyl cellulose, polyvinyl pyrrolidine, other cellulosic materials and starch), diluents (e.g. lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g. starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc).

Suitable transdermal delivery systems include patches, gels, tapes and creams, and can contain excipients such as solubilisers, permeation enhancers (e.g. fatty acids, fatty acid esters, fatty alcohols and amino acids), hydrophilic polymers (e.g. polycarbophil and polyvinyl pyrrolidine) and adhesives and tackifiers (e.g. polyisobutylenes, silicone-based adhesives, acrylates and polybutene).

Examples of transmucosal (notably rectal and intravaginal) delivery systems include patches, tablets, suppositories, pessaries, gels, and creams, and can contain excipients such as solubilizers and enhancers (e.g. propylene glycol, bile salts and amino acids), and other

vehicles (e.g. polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethyl cellulose and hyaluronic acid).

Injectable or implantable depot preparations may take the form of injectable fluids and implantation tablets. Suitable fluid carrier components are physiologically compatible diluents wherein the active agents can be dissolved, suspended. An example of a diluent is water, with or without addition of electrolyte salts or thickeners. Thus, the depot formulation can be, for example, an aqueous microcrystalline suspension. Oils are particularly suitable as diluents, with or without the addition of a solubiliser, of a surfactant, or of a suspension or emulsifying agent. Examples of suitable oils include arachidis oil, olive oil, peanut oil, cottonseed oil, soybean oil, castor oil, and sesame oil. Examples of solubilisers include benzyl alcohol and benzyl benzoate. Depot preparations offer the advantage that a single injection or implantation suffices for one or several months. Duration of the depot effect depends the nature of the estrogenic component (the ester precursors being preferred as they display a slower release), the amount of the estrogenic component as well as on the type of carrier substance that releases the active agent. Generally, the duration will be in the range of 10-30 days, but longer or shorter times can also be achieved.

Other delivery systems that can be used for administering the estrogenic components of the invention include intranasal and pulmonary delivery systems such as sprays and microparticles.

The invention is further illustrated by the following examples:

EXAMPLES

Example 1

Vaginal cornification was chosen as a tissue-specific and estrogen-sensitive endpoint to determine the estrogenicity of estetrol (E4), after both oral and subcutaneous administration, in hypoestrogenic rats. 17α-ethinylestradiol (EE), 17β-estradiol (E2) and vehicle (10% ethanol/sesame oil) served as controls in these bioassays.

Uterine weight increase in the rat is more commonly used as a measure of estrogenicity. However, uterine weight also responds to progesterone, testosterone, and other agents not characteristically regarded as estrogens. In the early 1920s it was discovered that follicular fluid from the pig ovary contained a factor(s) that caused cornification/keratinization of the vaginal epithelium in the rat (Allen and Doisy, 1923,

JAMA, 81, 819-821; Allen and Doisy, 1924, Am. J. Physiol., 69, 577-588). The so-called vaginal cornification response in rats subsequently provided a bioassay for testing estrogenicity. Vaginal epithelial cornification/keratinization in ovariectomized rats can be produced only by compounds considered to be true estrogens (Jones et al, 1973, Fert. Steril. 24, 284-291). Vaginal epithelial cornification/keratinization represents, therefore, a highly selective endpoint to determine the potency of estrogens (Reel et al., 1996, Fund. Appli. Toxicol. 34, 288-305).

Adult intact female CD rats were ovariectomized to induce estrogen deficiency. Vaginal lavages were performed daily for seven days to ensure that the rats demonstrated castrate vaginal smears (predominance of leukocytes in the vaginal smear, and similar in appearance to a diestrous vaginal smear). Castrate vaginal smears are indicative that complete ovariectomy was achieved. Treatment commenced following completion of the 7 days of smearing (day 0 = first day of dosing). Animals were dosed, once daily for 7 consecutive days. Daily vaginal lavages continued to be obtained for 7 days after dosing was initiated in order to detect vaginal cornification, as an indication of an estrogenic response. A drop of vaginal washings was placed on a glass slide and examined by light microscopy to detect the presence or absence of cornified epithelial cells. Vaginal lavages were obtained prior to dosing on days 0-6 and prior to necropsy on day 7.

The vaginal cornification bioassay was performed in order to determine the estrogenic profile of E4 when given subcutaneously (sc) to ovariectomized adult rats. E2 was used as a positive control. The vehicle (10% ethanol/sesame oil) served as the negative control. Steroids were dissolved in absolute ethanol and then brought to the final concentration with sesame oil (10% ethanol in sesame oil). A vaginal estrogenic response occurred in 8/8 rats by day 2 and persisted through day 7 in rats injected sc with 50 µg/kg/day E2 for 7 days (Table 1). Animals treated with the vehicle did not exhibit vaginal epithelial cornification (Table 1). The onset of vaginal epithelial cornification was dose-dependent in rats injected sc with 0.1, 0.3, 1.0, and 3.0 mg/kg/day E4 and started at the same day of treatment (Day 2) as observed for E2 (Table 1). At 0.1 mg/kg/day E4 already 4/8 rats and at 0.3 mg/kg/day E4 even 7/8 rats exhibited a vaginal estrogenic response by day 7. At 1.0 and 3.0 mg/kg/day E4 all rats showed a vaginal estrogenic response by day 7 (Table 1).

Table 1: Vaginal estrogenic response in ovariectomized rats treated subcutaneously (sc) with 17β-estradiol (E2) or estetrol (E4). Data are expressed as the number of rats showing vaginal cornification over the number of rats (ratio) treated.

Treatment Group	Dosing route	Number of Rats Exhibiting Estrogenic Response/ Number of Rats Treated							
		Day of Study							
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0.05 mg/kg/day E2	sc	0/8	0/8	8/8	8/8	8/8	8/8	8/8	8/8
Vehicle Control	sc	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
0.1 mg/kg/day E4	sc	0/8	0/8	0/8	1/8	1/8	4/8	3/8	4/8
0.3 mg/kg/day E4	sc	0/8	0/8	1/8	5/8	7/8	6/8	7/8	7/8
1.0 mg/kg/day E4	sc	0/8	0/8	1/8	6/8	8/8	7/8	8/8	8/8
3.0 mg/kg/day E4	sc	0/8	0/8	3/8	8/8	8/8	8/8	8/8	8/8

The vaginal cornification bioassay was performed in order to determine the estrogenic profile of E4 when given orally (po) to ovariectomized adult rats. EE was used as a positive control. The vehicle (10% ethanol/sesame oil) served as the negative control. Steroids were dissolved in absolute ethanol and then brought to the final concentration with sesame oil (10% ethanol in sesame oil). A vaginal estrogenic response occurred in all rats (8/8) given 50 µg/kg/day EE po by day 7 (Table 2). Similarly, vaginal epithelial cornification was observed in all rats (8/8) treated po with either 0.1, 0.3, 1.0, or 3.0 mg/kg/day E4 by day 7 (Table 2), whereas animals treated with the vehicle did not exhibit vaginal epithelial cornification (0/8). Surprisingly, even in rats given relatively low doses of E4 (e.g. 0.1 mg/kg/day), the onset of vaginal cornification (defined as the amount of animals responding at days 1-3 of the study) was faster in po-treated than in sc-treated animals, demonstrating estetrol's superb bioavailability characteristics after oral administration.

Table 2: Vaginal estrogenic response in ovariectomized rats treated orally (po) with 17α-ethinyl estradiol (EE) or estetrol (E4). Data are expressed as the number of rats showing vaginal cornification over the number of rats (ratio) treated.

Treatment Group	Dosing route	Number of Rats Exhibiting Estrogenic Response/ Number of Rats Treated							
		Day of Study							
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0.05 mg/kg/day EE	po	0/8	1/8	3/8	8/8	8/8	8/8	8/8	8/8
Vehicle Control (2 ml/kg/day)	po	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
0.1 mg/kg/day E4	po	0/8	0/8	1/8	7/8	8/8	8/8	8/8	8/8
0.3 mg/kg/day E4	po	0/8	0/8	1/8	7/8	8/8	8/8	8/8	8/8
1.0 mg/kg/day E4	po	0/8	0/8	4/8	8/8	8/8	8/8	8/8	8/8
3.0 mg/kg/day E4	po	0/8	0/8	6/8	8/8	8/8	8/8	8/8	8/8

Example 2

To evaluate the oral (po) and subcutaneous (sc) bioavailability of estetrol (E4) and to determine the elimination half-life, single dose studies were performed in female Sprague Dawley rats followed by frequent blood sampling over a 24 hours interval.

Female Sprague Dawley rats were equipped with a permanent silatic heart catheter, as described by Kuipers et al. (1985, Gastroenterology, 88, 403-411). Rats were allowed to recover from surgery for 5 days and were than administered 0.05, 0.5, or 5 mg/kg E4 in 0.5 ml arachidis oil. For sc administration, E4 was injected in the neck area using a 1 ml syringe and 20g needle. For po administration of E4, rats were lightly anaesthesized with halothene/ N_2O/O_2 and E4 was directly applied intragastrically using a plastic stomach intubator. Blood samples were subsequently collected via the heart catheter in heparinized tubes at 0.5, 1, 2, 4, 8 and 24 hours. Erythrocytes were removed by centrifugation at 5000xg for 10 minutes at 4°C and blood plasma was stored at –20°C. After thawing the plasma samples, liquid-liquid extraction (hexane and diethyl ether) was employed to prepare the E4-containing plasma samples for HPLC analysis (Perkin Elmer 200) and tandem mass spectrometry using a PE

Sciex 3000 tandem mass spectrometer and APCI interface. With each sample batch, a calibration curve with 6 calibrators was recorded. The calibration curve was calculated using linear regression (correlation coefficient > 0.98), which permitted quantitation of plasma concentrations. For each rat plasma, sampled at different time intervals, data were collected.

Plasma E4 concentration data were analysed with "WinNonLin, edition 3.1" and involved pharmacokinetic parameters for C_{max}, half-life and AUC_{0-24}. Especially, using the lower and intermediate dose levels of 0.05, 0.5 mg/kg, E4 demonstrated an oral bioavailability equal to the bioavailability obtained with sc administration (80-100 %). At the highest dose level tested, 5.0 mg/kg E4, absorption kinetics gave rise to an oral bioavailability approximating 30-60% of sc administered E4. Interestingly, E4 demonstrated a relatively long half-life of 2-3 hours, enabling the detection of bioactive levels of unconjugated E4 at all time points over a 24 hour interval in the sc and po dosing experiments.

Example 3

To determine the bioavailability and elimination half-life of estetrol after oral dosing in humans a single rising dosing study was performed in healthy postmenopausal volunteers. Volunteers (n=6) were randomly assigned to 0.1, 1 or10 mg estetrol and blood samples (18 per volunteer) were obtained over a period of 72 hours.

After thawing the plasma samples, liquid-liquid extraction (hexane and diethyl ether) was employed to prepare the estetrol-containing plasma samples for HPLC analysis (Perkin Elmer 200) and tandem mass spectrometry using a PE Sciex 4000 tandem mass spectrometer and APCI interface. With each sample batch, a calibration curve with 6 calibrators was recorded. The calibration curve was calculated using linear regression (correlation coefficient > 0.98), which permitted quantitation of plasma concentrations.

Good tolerability was observed when increasing the oral estetrol dose from 0.1 to 1 and further to 10 mg. AUC values demonstrated good dose-linearity, indicating that, over the entire dose range, orally administered estetrol was well absorbed. Interestingly, estetrol demonstrated a long elimination half-life of more than 15 hours, i.e.15-50 hours in human postmenopausal subjects.

Example 4

Established competitive steroid binding assays were used to determine the relative binding affinity of estetrol (E4), as compared to 17α-ethinylestradiol(EE) and 17β-estradiol (E2), to human Estrogen Receptor (ER) α- and β-forms.

The method employed was adapted from the scientific literature and described in detail by Osbourn et al. (1993, Biochemistry, 32, 6229-6236). Recombinant human ERα and ERβ proteins were purified from transfected Sf9-cells. The *in vitro* assays involved the use of either ERα or ERβ proteins and [^{3}H]E2, at a fixed concentration of 0.5 nM, as the labeled ligand. Recombinant human ERα or ERβ proteins were dissolved in binding buffer (10 mM Tris-HCL, pH 7.5, 10% glycerol, 1 mM DTT, 1 mg/ml BSA) and duplicate aliquots were then incubated with [^{3}H]E2 at a final concentration of 0.5 nM, together with a vehicle control (0.4% DMSO), or the same amount of vehicle containing increasing concentrations of unlabeled steroid ligands as competitors. After incubation for 2 h at 25^{0}C, the unbound ligands were removed and the amounts of [^{3}H]E2 bound to either ERα or ERβ proteins were measured. The average amounts of [^{3}H]E2 bound to either ERα or ERβ proteins at each concentration of competitor were used to make inhibition curves. IC50 values were subsequently determined by a non-linear, least squares regression analysis. Inhibition constants (Ki) were calculated using the equation of Cheng and Prusoff (Cheng et al., 1973, Biochem. Pharmacol., 22, 3099-3108), using the measured IC50 of the tested compounds, the concentration of radioligand employed in the assay, and the historical values for the Kd of the radioligand, which were established as 0.2 nM and 0.13 nM for ERα and ERβ, respectively.

Biochemical assay results for E4 are presented as the percent inhibition of specific binding in three separate experiments (Table 3). For comparision of binding affinities of E4, EE and E2 to human ERα and ERβ proteins, experimentally observed Ki values are shown in Table 4. As compared to EE and E2, E4 demonstrates a unique binding profile with a strong preference (400%) for binding to the ERα protein (Table 4). In contrast, Ki values for ERβ protein are more pronounced for EE and E2 steroid ligands (Table 4).

Table 3: Percent inhibition of specific binding to ERα and ERβ proteins using E4 as unlabeled steroid ligand and 0.5 nM [3H] as labeled competitor. Results of three separate experiments are shown.

E4 final concentration	Percent inhibition of specific binding in					
	ERα steroid binding assay			ERβ steroid binding assay		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
1 μM	98	nd	nd	87	90	95
0.3 μM	92	94	101	74	74	77
0.1 μM	83	85	86	56	54	50
0.03 μM	64	66	63	19	25	30
10 nM	43	32	28	nd	nd	nd
3 nM	26	17	11	nd	nd	nd

nd: not determined

Table 4: Experimentally determined inhibition constants (Ki) for estetrol (E4), 17α-ethinylestradiol (EE) and 17β-estradiol (E2), to human ERα and ERβ proteins. Relative preference for binding to ERα protein is also shown.

Steroid ligands	Ki ERα (nM)	Ki ERβ (nM)	Relative ERα/ERβ preference(%)
EE	0.23	0.025	11
E2	0.21	0.015	7
E4	4.9	19	400

Example 5

An established competitive steroid-binding assay (Hammond and Lahteenmaki. 1983. Clin Chem Acta 132:101-110) was used to determine the relative binding affinity of estetrol (E4), 17α-ethinylestradiol(EE2), 17β-estradiol (E2), testosterone (T)and 5α-dihydrotestosterone (DHT) for human sex Hormone Binding Globulin (SHBG).

Human SHBG was purified from transgenic mouse serum, as described previously (Avvakumov GV et al., 2000. J Biol Chem 275: 25920-25925). The human SHBG prepared in this way was assessed to be >99% pure by polyacrylamide gel electrophoresis under

denaturing conditions. Its steroid-binding characteristics are indistinguishable from SHBG in human serum (Avvakumov GV et al., 2000. J Biol Chem 275: 25920-25925). The *in vitro* assay involved the use of the purified human SHBG and [^{3}H]DHT or [^{3}H]estradiol as labeled ligands. Human SHBG was treated for 30 min at room temperature with a dextran-coated charcoal (DCC) suspension in phosphate buffered saline (PBS) to remove any steroid ligand. After centrifugation (2,000 x g for 10 min) to sediment the DCC, the supernatant containing the human SHBG was diluted in PBS to a concentration of 1 nM based on its steroid binding capacity.

Duplicate aliquots (100 μl) of this human SHBG solution were then incubated with an equal volume of either [^{3}H]DHT or [^{3}H]estradiol at 10 nM, together with 100 μl of PBS alone or the same amount of PBS containing increasing concentrations of unlabeled steroid ligands as competitors in polystyrene test tubes. After incubation for 1 h at room temperature the reaction mixtures were placed in an ice bath for a further 15 min. Aliquots (600 μl) of an ice cold suspension of DCC were then added to each tube, and after a brief 2 seconds mixing, each tube was incubated in an ice bath for either 10 min or 5 min depending on whether [^{3}H]DHT or [^{3}H]estradiol were being used as labeled ligands, respectively. The unbound ligands adsorbed to DCC were then removed by centrifugation (2, 000 x g for 15 min at 4 C), and the amounts of [^{3}H]labeled ligands bound to SHBG were counted in 2 ml ACS scintillation cocktail using in liquid scintillation spectrophotometer. The average amounts of [^{3}H]labeled ligands bound to SHBG at each concentration of competitor (B) were expressed as a percentage of the average amounts of [^{3}H]labeled ligands bound to SHBG in the absence of competitor (B_0), and were plotted against the concentration of competitor in each assay tube. The results of the competitive binding assays are depicted in Figure 1.

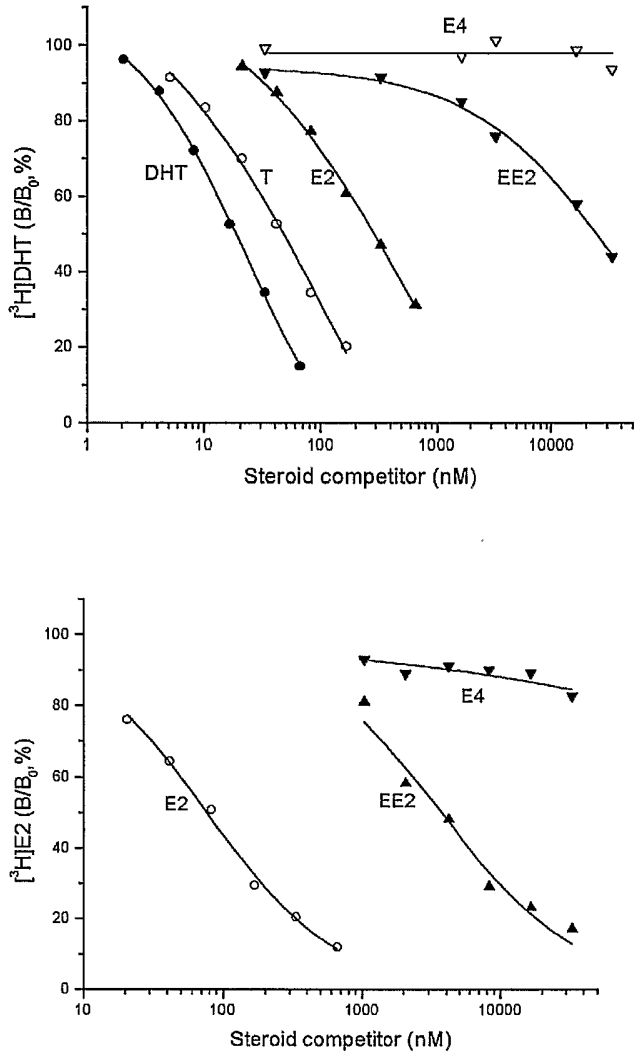


Figure 1: Competitive displacement of [^{3}H]DHT (panel A) and [^{3}H]estradiol (panel B) from the human sex hormone-binding globulin steroid binding site. The unlabeled steroid ligands used as competitors were as follows: estetrol (E4), 17α-ethinylestradiol (EE2), 17β-estradiol (E2), testosterone (T) and 5α-dihydrotestosterone (DHT)

As is clearly apparent from these competitive binding assays, estetrol does not bind at all to human SHBG when tested with either [^{3}H]DHT or [^{3}H]estradiol as labeled ligands. This is in marked contrast with reference steroids ethinylestradiol, 17β-estradiol, testosterone and 5α-dihydrotestosterone, which, in this order, show an increased relative binding affinity for human SHBG. Importantly, estetrol binding to SHBG was negligible when compared with the other estrogens tested, ethinylestradiol and 17β-estradiol.

Example 6

A study is conducted to investigate the serum lipid effects of estetrol therapy in postmenopausal women.

Twenty women are allowed to enter the study, if the following conditions are met:

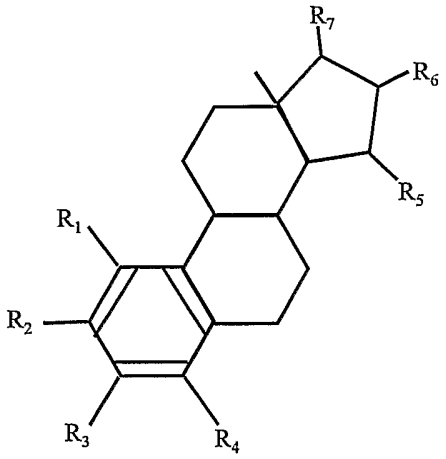
- postmenopausal;
- without pre-existing cardiovascular disease(s);
- having a low-density lipoporotein cholesterol (LDL-C) plasma level in excess of 150 mg/dL;
- not using hormone replacement therapy or estrogen replacement therapy or other plasma lipid levels influencing therapy (e.g. statins).

The plasma levels of LDL-C, VLDL-C, HDL-C, triglycerides (TC), Lp(a), apolipoprotein A1, A2, B and total cholesterol are measured on the date of entry of the study (= 0 weeks) and after 4 and 8 weeks. If LDL-C levels are found to remain over 150 mg/dL, participants are allowed to participate in an intervention therapy which is started immediately after the first 8 weeks.

Ten volunteers use orally 2mg/day estetrol, for a period 3 months. During this therapy period, LDL-C, VLDL-C, HDL-C, TC, Lp(a), apolipoprotein A1, A2, B and total cholesterol (TC) are measured every 4 weeks (i.e. in week 12 and 16).The levels of LDL-C, VLDL-C, HDL-C, TC, Lp(a), apolipoprotein A1, A2, B and total cholesterol of week 16 are compared with the same levels of week 8. The LDL-C level is found to have decreased significantly. The observed changes in levels of VLDL-C, HDL-C, TC, Lp(a), apolipoprotein A1, A2, B and total cholesterol also show a favourable trend

CLAIMS

1. Use of an estrogenic component selected from the group consisting of:
substances represented by the following formula



in which formula R_1, R_2, R_3, R_4 independently are a hydrogen atom, a hydroxyl group or an alkoxy group with 1-5 carbon atoms; each of R_5, R_6, R_7 is a hydroxyl group; no more than 3 of R_1, R_2, R_3, R_4 are hydrogen atoms;

precursors capable of liberating a substance according to the aforementioned formula when used in the present method; and

mixtures of one or more of the aforementioned substances and/or precursors;

in the manufacture of a pharmaceutical composition for use in a method of treating or preventing a cardiovascular pathology in a mammal, said method comprising the administration of a therapeutically effective amount of the estrogenic component to said mammal.

2. Use according to claim 1, wherein R_3 represents a hydroxyl group or an alkoxy group.

3. Use according to claim 1 or 2, wherein at least 3 of the groups R_1, R_2, R_3 and R_4 represent hydrogen atoms.

4. Use according to any one of claims 1-3, wherein the precursors are derivatives of the estrogenic substances wherein the hydrogen atom of at least one of the hydroxyl groups has been substituted by an acyl radical of a hydrocarbon carboxylic, sulfonic acid or sulfamic acid

of 1-25 carbon atoms; tetrahydrofuranyl; tetrahydropyranal; or a straight or branched chain glycosydic residue containing 1-20 glycosidic units per residue.

5. Use according to any one of claims 1-4 , wherein the method comprises the uninterrupted administration of the estrogenic component during a period of at least 5 days, preferably of at least 30 days.

6. Use according to any one of claims 1-5, wherein the method comprises oral, intravenous or subcutaneous administration of the estrogenic component.

7. Use according to claim 6, wherein the method comprises oral administration.

8. Use according to any one of claims 1-7, wherein the estrogenic component is administered in an amount of at least 1 μg per kg of bodyweight per day, preferably of at least 5 μg per kg of bodyweight per day.

9. Use according to any one of claims 1-8, wherein the cardiovascular pathology is selected from the group consisting of hypercholesterolemia, dyslipidemia, atherosclerosis, arteriosclerosis, xanthomatosis, myocardial infarction, stroke, multiple silent stroke, unstable angina, loss of cognitive function, vascular dementia and Alzheimer disease.

10. Use according to any one of claims 1-9, wherein the cardiovascular pathology is selected from the group consisting of hypercholesterolemia and atherosclerosis.

11. Use according to any one of claims 1-10, wherein the mammal's total blood cholesterol level exceeds 240 mg/dL and/or blood LDL-cholesterol level exceeds 100 mg/dL and/or blood high density lipoprotein cholesterol (HDL-C) is below 35 mg/dL and/or blood triglycerides level exceeds 150 mg/dL.

12. Use according to any one of claims 1-11, wherein the method comprises administering the estrogenic component in an amount effective to reduce the mammal's serum level of low density lipoprotein cholesterol (LDL-C) and/or apolipoprotein B.

13. Use according to any one of claims 1-12, wherein the method comprises administering the estrogenic component in an amount effective to reduce the mammal's serum level of Lp(a).

14. Use according to any one of claims 1-13, wherein the method comprises administering the estrogenic component in an amount effective to increase the mammal's serum level of HDL-C and/or apolipoproten A1.

15. Use according to any one of claims 1-14, wherein the method comprises administering the estrogenic component in an amount effective to prevent *in vivo* oxidation of low density lipoprotein.

INTERNATIONAL SEARCH REPORT

Internation plication No
PCT/NL 03/00421

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/565 A61P9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SEEGER, H. ET AL: "The inhibitory effect of endogenous estrogen metabolites on copper-mediated in vitro oxidation of LDL" INTERNATIONAL JOURNAL OF CLINICAL PHARMACOLOGY AND THERAPEUTICS, vol. 36, no. 7, 1998, pages 383-385, XP001095070 table 1 page 384, column 1, paragraph 4 - page 385, column 1, line 2 ----- -/--	1-15

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

° Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 October 2003	22/10/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Giacobbe, S.

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	SUBBIAH M T R: "Estrogen replacement therapy and cardioprotection: Mechanisms and controversies." BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH, vol. 35, no. 3, March 2002 (2002-03), pages 271-276, XP001095274 ISSN: 0100-879X the whole document	1-15
Y	ZUCKERMAN STEVEN H ET AL: "Inhibition of LDL oxidation and myeloperoxidase dependent tyrosyl radical formation by the selective estrogen receptor modulator raloxifene (LY139481 HCL)." ATHEROSCLEROSIS, vol. 126, no. 1, 1996, pages 65-75, XP002208776 ISSN: 0021-9150 page 65, column 2, paragraph 1 - page 66, column 2, paragraph 1	1-15
Y	LIPPERT CAROLINE ET AL: "The effects of A-ring and D-ring metabolites of estradiol on the proliferation of vascular endothelial cells" LIFE SCIENCES, PERGAMON PRESS, OXFORD, GB, vol. 67, no. 13, 18 August 2000 (2000-08-18), pages 1653-1658, XP002200988 ISSN: 0024-3205 table 1 page 1657, paragraph 1	1-15
Y	MUECK, A. O. ET AL: "Angiogenetic and anti-angiogenetic effects of estradiol and its metabolites" JOURNAL OF CLINICAL AND BASIC CARDIOLOGY, vol. 4, no. 2, 2001, pages 153-155, XP0008004903 the whole document	1-15
Y	MOORADIAN ARSHAG D: "Antioxidant properties of steroids." JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY, vol. 45, no. 6, 1993, pages 509-511, XP001095273 ISSN: 0960-0760 the whole document	1-15

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INESTROSA N C ET AL: "CELLULAR AND MOLECULAR BASIS OF ESTROGEN'S NEUROPROTECTION POTENTIAL RELEVANCE FOR ALZHEIMER'S DISEASE" MOLECULAR NEUROBIOLOGY, HUMANA PRESS, US, vol. 17, no. 1-3, 1998, pages 73-86, XP001010530 ISSN: 0893-7648 the whole document	1-15
Y	BRINTON R D ET AL: "THE ESTROGEN REPLACEMENT THERAPY, PREMARIN, INCREASES NEURONAL SURVIVAL AND PROTECTS NEURONS AGAINST OXIDATIVE DAMAGE: IMPLICATIONS FOR ALZHEIMER'S DISEASE" SOCIETY FOR NEUROSCIENCE ABSTRACTS, SOCIETY FOR NEUROSCIENCE, US, vol. 1/2, no. 23, 1997, page 28,AN2005, XP001068253 ISSN: 0190-5295 the whole document	1-8