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Untargeted search and identification of metabolites of antiviral agent camphecene in rat urine by liquid chromatography and mass spectrometry and studying their distribution in organs following peroral administration of the compound

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ABSTRACT

Major metabolites of camphecene, a new effective antiviral agent, formed after its oral administration to rats and excreted in the urine, were found and identified using liquid chromatography coupled to mass spectrometry as well as multivariate analysis of HPLC-MS data. The metabolites were found to be camphecene glucuronide, camphecene sulfate and the corresponding iminoacid. A study of the dynamics of accumulation of camphecene and its metabolites in the liver, kidneys, lungs and brain of animals was performed. Maximum concentration of camphecene in blood and organs was reached after 1.5–2 h of its administration, and the maximal content of the agent in the organs investigated was observed in the kidneys. The content of the substance in the lungs was comparable to that in the liver. Also, camphecene was found in brain in high concentration, thus allowing assumption of its ability to penetrate the blood-brain barrier and to exert its antiviral properties in the organ. Camphecene glucuronide and iminoacid had concentration-time profiles similar to that of their precursor, their content being maximal in kidney and liver and 2–3 orders of magnitude higher than in lungs and brain. The content of camphecene sulfate was of similar level in all organs studied. The results obtained made it possible to develop recommendations for therapy with the use of camphecene.

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1. Introduction

Currently, drugs with different mechanisms of pharmacological action are available for the prevention and treatment of influenza infection. Internationally recognised etiotropic anti-influenza drugs are chemical compounds of two mechanisms of action – M2 channel blockers, such as adamantane derivatives (amantadine and its analogue in Russia, remantadine) [1], and inhibitors of viral neuraminidase – oseltamivir (Tamiflu) and zanamivir (Relenza) [2]. The drawback of the drugs of both the first and the second group is the ability of the virus quickly to develop resistance. Significant efforts of our group have been applied to the search for new agents with a wide range of antiviral activity. We used available bicyclic monoterpenes of framework structure as the initial structural blocks for the synthesis of target antiviral agents. Due to the inherent characteristics of natural substances in general, and of terpenoids in particular, i.e. complementarity to many targets in the body, terpenoids are a unique base for the design of new biologically active compounds, including antiviral agents [3]. An important advantage of using terpenoids as starting compounds is their availability in both enantiomeric forms with high optical purity, which makes it possible to create highly selective agents for binding to asymmetric natural targets. A number of our synthesised camphor and borneol derivatives showed outstanding antiviral activity, in particular against the influenza virus [4] and filoviruses [5]. The product of the interaction of camphor and aminoethanol, 2-((1R,4R)-1,7,7-trimethylbicyclo [2,2,1]heptan-2-ylidene)aminoethanol, which was given the name camphecene

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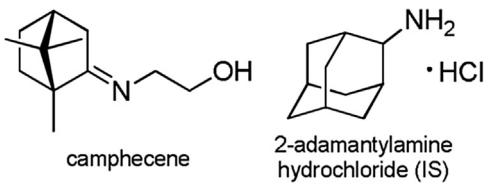

Fig. 1. Chemical structures of camphecene and 2-adamantylamine hydrochloride (2-Ad, internal standard).

(Fig. 1), showed high activity against influenza virus strains A H1N1, H3N2, H5N2 and influenza B virus, acting in the early stages of viral replication [6]. As part of preclinical research, we developed and validated a method for quantitative analysis of camphecene in rat blood and plasma and determined the main pharmacokinetic parameters for intravenous administration of the substance [7,8].

An extremely important aspect in the development of a drug, apart from assessing its pharmacokinetic parameters, is the identification of its main metabolite(s). Being administrated *per os*, xenobiotics often undergo the first-pass metabolism which occurs mainly in the liver or in the gut [9]. As a result, the fraction of free circulating compound is reduced that may require increasing the dosage of the drug, and the metabolites which are formed enter the blood stream and then are excreted.

With regard to drugs, it should be borne in mind that in a number of cases it is the metabolic products, and not the original substance, that determine its pharmacological properties. For example, the anti-influenza drug Tamiflu (oseltamivir phosphate), in order to become an effective antiviral agent, must be metabolised in the liver [10]. Also, the antiviral compound of a wide spectrum of activity, favipiravir (T-705, Avigan), is first converted into its ribose-5′-monophosphate followed by phosphorylation to corresponding triphosphate. The latter is a substrate for the viral polymerase, which joins the daughter RNA chain and provides a lethal mutagenesis in the viral population [11].

The search of metabolites of a drug requires utilisation of sensitive equipment that usually includes a chromatograph for separation of compounds and a detector for their identification. GC/MS is well suited for the detection of volatile compounds, while this method is not appropriate for screening non-volatile or thermolabile substances. HPLC with UV detection doesn't provide any information on the molecular weight of a compound and can be used for the search of compounds absorbing in UV diapason. Most appropriate and therefore most often used method for the search and identification of unknown substances is HPLC coupled with mass spectrometric detection.

In this work, we searched for and identified the main metabolites of camphecene that are formed following its oral administration to rats and excreted in the urine, and also investigated the dynamics of accumulation of camphecene in blood and in the liver, kidneys, lungs and brain and its metabolites in the organs within 24 h of a single dosage of the substance.

2. Experimental

2.1. Chemicals and reagents

Camphecene was synthesised according to the procedure described earlier [12] and purified by double vacuum distillation. The purity of the compound was over 99% according to GC/MS. 2-Adamantylamine hydrochloride (2-Ad, Fig. 1) used as internal standard (IS) was purchased from Sigma-Aldrich. Analytical grade methanol was purchased from Merck (Darmstadt, Germany), zero-grade acetonitrile (ACN) was purchased from Cryochrom (Saint-Petersburg, Russia). Formic acid was purchased from Panreac (Barcelona, Spain). High purity water was prepared using a Direct-Q 3 UV system (Millipore S. A. S., France). Whatman Protein Saver 903 Cards were purchased from Sigma-Aldrich and used for dried blood spot sampling. The QuEChERS vetexQ Tox extraction kit was purchased from InterLab Ltd. (Moscow, Russia).

2.2. Compound administration, urine sample collection and preparation for analysis

A group of five animals participated in the experiment. Blank urine samples were taken from each animal and immediately frozen. Camphecene at a dose of 100 mg/kg of body weight was administrated *per os* to each animal. After administration of the substance, spontaneously excreted urine was collected from the animals at 3, 6, 7, 8 and 24 h. All urine samples were frozen and kept at −70 °C.

Prior to sample preparation, the urine was thawed and brought to ambient temperature. A 20 μl sample of the urine was diluted with 980 μl of water and vortexed for 20–30 s. A 20 μl aliquot of the obtained solution was mixed with 1780 μl of water and 200 μl of ACN containing 2-Ad at a concentration of 1000 ng/ml and vortexed for 20–30 s. The sample obtained was used for the analysis.

2.3. Compound administration, collection of blood and organ samples

The work was performed on mature Wistar rats obtained from the Animal Facility of the Institute of Cytology and Genetics of the SB RAS, in compliance with the principles of the Helsinki Declaration on Humane Treatment of Animals. In the work, males weighing 220–250 g were used. The animals were kept in standard conditions with free access to tap water and dry food in individual cages. Rats from a breeding colony were housed in groups, 4–6 per cage. The housing conditions were standard at 12:12 h light-dark cycle. Animals received tap water *ad libidum*, their pellet chow consisted of 0.3% sodium, 0.8% potassium, and 20% protein. A rat was placed alone in a metabolic cage (Techniplast, Italy) a day before and during performance of the experiments. During the experiment, the feed was removed from 0 to 8 h inclusive; from 8 to 24 h, feed was available to the animals.

The experiments were performed on a group of 14 animals, which were given camphecene *per os* at a dose of 100 mg/kg. An individual weighing of the substance was mixed with 40 μl of Tween-80, and physiological saline was added for dilution to a volume of 0.2 ml per 100 g of rat mass. The control group received Tween-80 in physiological saline.

Samples of blood and organs were taken 1, 2, 3, 4, 6, 8 and 24 h after administration of camphecene; two animals were taken for sampling. Aliquots of 20 μl of blood was taken from the tail vein in duplicate and spotted onto the Whatman Protein Saver 903 Cards. The samples were dried for 2 h in air and then stored at + 4 °C until analysis. Immediately after blood sampling, the animals were anaesthetised with an i.p. injection of sodium thiopental (10 mg/kg bw), and, following decapitation, the kidneys, liver, lungs and brain were excised and snap-frozen in liquid nitrogen. The organs were kept at −80 °C and brought to ambient temperature prior to sample preparation.

2.4. Sample preparation of organs for LC–MS/MS analysis

Dried blood spot samples were processed in accordance with the method developed and validated earlier [7].

Sample preparation of organs was carried out in accordance with a protocol described by Yuan et al. [13], three samples were taken from each organ for the analysis. Briefly, a sample of an organ weighing about 10–15 mg (exact weight) was thawed and

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