

QUALITATIVE GC-MS ASSESSMENT OF TCP AND TAMORF ELIMINATION IN RATS

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Nerve agents are highly toxic organophosphorus (OP) compounds. They inhibit acetylcholinesterase (AChE), an enzyme that hydrolyses acetylcholine (ACh) in the nervous system. Pathophysiological changes caused by OP poisonings are primarily the consequence of surplus ACh on cholinergic receptors and in the central nervous system. Standard treatment of OP poisoning includes combined administration of carbamates, atropine, oximes and anticonvulsants. In order to improve therapy, new compounds have been synthesised and tested. Tenocyclidine (TCP) and its adamantane derivative 1-[2-(2-thienyl)-2-adamantyl]morpholine (TAMORF) have shown interesting properties against soman poisoning. In this study, we developed a qualitative GC-MS method to measure elimination of TCP and TAMORF through rat urine in order to learn more about the mechanisms through which TCP protects an organism from OP poisoning and to determine the duration of this protective effect. GC-MS showed that six hours after treatment with TCP, rat urine contained only its metabolite 1-thienylcyclohexene, while urine of rats treated with TAMORF contained both TAMORF and its metabolites.

KEY WORDS: *antidote, metabolite, qualitative method, urine, tenocyclidine*

Organophosphorous (OP) compounds are highly toxic substances widely used in agriculture as pesticides. Very few of them are prescribed to treat cholinergic disorders in humans (1-4). The most toxic organophosphates sarin, soman, tabun, and VX have long been used as nerve agents in chemical warfare and terrorist actions (1, 2, 5, 6). Due to irreversible inhibition of the enzyme acetylcholinesterase (AChE), OP poisoning primarily leads to extracellular accumulation of neurotransmitter acetylcholine (ACh) at muscarinic receptors of the peripheral and central nervous system, causing a host of symptoms including hypersalivation, lacrimation, diarrhoea, tremor, respiratory distress, epileptiform convulsions, and heart failure. Signs are dose-dependent, leading to severe incapacitation and rapid death (1, 2, 7-9). The current standard treatment of OP poisoning includes pre-treatment with reversible AChE-inhibitors such

as carbamate pyridostigmine bromide or combined therapy with oximes (cholinesterase reactivators), atropine sulphate (a muscarinic cholinergic receptor antagonist), and benzodiazepines (anticonvulsants). However, standard treatment leaves much to be desired since none of the standard oximes can be regarded as a universally suitable reactivator of AChE. In case of soman poisoning, reinhibition of AChE is faster than reactivation, and oxime therapy is ineffective due to the "aging" of the enzyme, which makes reactivation impossible (2, 10-13).

To improve therapy against OP poisoning, new compounds have been synthesised and tested. Tenocyclidine (TCP, Figure 1), derivative of phencyclidine (PCP), has proven itself as an effective adjuvant therapy to atropine, diazepam, and HI-6 (oxime) against soman poisoning in guinea pigs (1, 14).

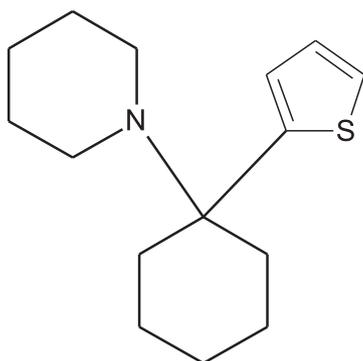


Figure 1 Structural formula of tenocyclidine (TCP),
1-[1-(2-thienyl)cyclohexyl]piperidine

It seems that the therapeutic effect of TCP is related to the excitatory amino acid glutamate neurotransmission and especially to N-methyl-D-aspartate (NMDA) receptors, which, just like muscarinic receptors, are involved in soman-induced seizures (1,14-16). However, what limits TCP use is its toxicity (2). Škare et al. (1) used three adamantyl TCP derivatives, 1-[2-(2-thienyl)-2-adamantyl] piperidine (TAPIP), 1-[2-(2-thienyl)-2-adamantyl] pyrrolidine (TAPIR), and 1-[2-(2-thienyl)-2-adamantyl] morpholine (TAMORF), assuming that replacing the cyclohexyl group of TCP with an adamantyl group and piperidine, pyrrolidine, or morpholine would reduce TCP toxicity and improve its pharmacological properties. When adamantyl derivatives were administered with atropine and HI-6 to mice poisoned with soman, TAMORF (Figure 2) showed the best antagonising effect (1). Pretreatment with TAMORF protected animals from the lethal effects of soman and eliminated most of the soman-induced signs of toxicity in poisoned rats (2). Both TCP and TAMORF have shown low genotoxicity (2, 17, 18). Besides antidotal efficiency, TCP and TAMORF showed interesting radioprotective properties (17).

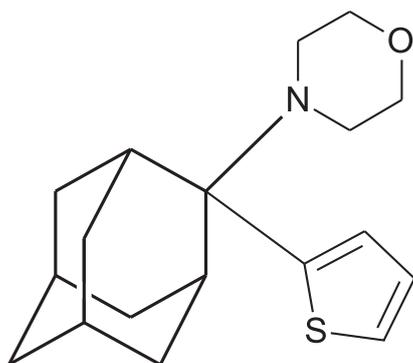


Figure 2 Structural formula of TAMORF,
1-[2-(2-thienyl)-2-adamantyl] morpholin

The aim of this study was to learn more about the pathways through which TCP and its adamantyl derivatives protect against OP poisoning. For this purpose we developed a gas chromatography - mass spectrometry (GC-MS) method to establish TCP and TAMORF elimination through urine in rats six hours after treatment, as this information speaks about the duration of their antidotal activity.

MATERIALS AND METHODS

Pure TCP and TAMORF

TCP and TAMORF were prepared according to a procedure reported earlier (1). They were identified and their purity determined at Ruđer Bošković Institute (Zagreb, Croatia) using spectroscopic techniques (infrared spectroscopy, ^1H and ^{13}C nuclear magnetic resonance and mass spectrometry) and elemental analysis.

Mass spectra of TCP and TAMORF powders were scanned for later comparison with the mass spectra of signals from the GC-MS chromatogram.

Mass spectrometer and operational conditions

The instrument consisted of a quadrupole MS (QP-2010, Shimadzu, Japan), PC, and a printer. Ionization mode was electron impact (EI), ion source temperature $200\text{ }^\circ\text{C}$, inlet temperature range $40\text{ }^\circ\text{C}$ to $200\text{ }^\circ\text{C}$ with the rate of $20\text{ }^\circ\text{C min}^{-1}$; the acquisition mode was scan and the scanning rate was 40.00 m/z to 550.00 m/z .

Standard chloroform solutions of TCP and TAMORF

2.00 mg of TCP or TAMORF was added to 1.00 mL of chloroform (p. a., Merck, Germany), and shaken in an ultrasonic bath (Branson 5210, Branson, USA) for 10 min. The solutions were then filtered (Chromafil RC 45/15 MS, Macherey-Nagel, Germany) into analytical vials and analysed with GC-MS.

Extraction of TCP and TAMORF from spiked rat urine samples

2.00 mg of TCP was added to 1 mL of deionised water, and 2.00 mg of TAMORF was added to 1 mL of glycerol formal (*purum grade*, Fluka, Switzerland). Solutions were shaken in an ultrasonic bath (Branson 5210, Branson, USA) for 10 min. An aliquot was

added to 1.5 mL of rat urine, and the mixtures shaken in the ultrasonic bath (Branson 5210, Branson, USA) for another 10 min. One millilitre of chloroform was then added to urine solutions, and the mixtures centrifuged (EBA, Hettich, USA,) for 5 min at 2500 rpm. Chloroform phases were put into analytical vials and analysed with GC-MS.

Animals

Male adult Wistar rats were obtained from the Institute for Medical Research and Occupational Health (Zagreb, Croatia) and selected by mass (240 g to 280 g). The animals were kept in macrolone cages under controlled conditions (room temperature 21 °C, 12 h : 12 h light:dark cycle), received a standard diet for laboratory rodents (Sljeme, Zagreb, Croatia), and had free access to water. The rats were randomly distributed in groups of four and were deprived of food for 24 h before the experiment. The study was carried out according to the NIH Guide for the Care and Use of Laboratory Animals (19). The experiments were approved by the Institute's ethics committee and by the Ministry of Science, Education and Sports of the Republic of Croatia.

Treatment of animals

The first group was intraperitoneally (*i.p.*) administered TCP dissolved in deionised water in the concentration of 2.5 mg kg⁻¹ body mass. The second group (negative control - water) received an *i.p.* dose of deionised water (in a volume corresponding to that of the TCP solution administered to the first group). The third group received an *i.p.* dose of TAMORF dissolved in glycerol formal in the concentration of 125 mg kg⁻¹ body mass. The fourth group (negative control - saline) received an *i.p.* dose of saline (in a volume corresponding to that of the TAMORF solution administered to the third group). The fifth group (negative control - solvent) received an *i.p.* dose of glycerol formal (in a volume corresponding to that of the TAMORF solution administered to the third group).

Urine collection and analysis

Rat urine was collected over six hours after treatment and stored at 4 °C till analysis.

One millilitre of chloroform was added to 1.5 mL of collected urine and the mixture centrifuged at 2500 rpm for 5 min. Chloroform phases were put into analytical vials and analysed with GC-MS.

GC-MS system and operational conditions

The GC-MS system consisted of a gas chromatograph (Agilent Technologies 6890N Network GC System, USA), an Agilent 19091S-433 column (HP 5 MS, 30 m x 0.25 mm; 0.25 µm film thickness), helium as carrier gas (Helium 5.0, Messer, Austria), mass selective detector (Agilent Technologies 5973 Network), a PC, and a printer. The initial temperature of the GC oven was 90 °C (for 6.00 min) and the final temperature was 295 °C (for 10.00 min). The temperature rate was 15 °C min⁻¹, injector temperature 295 °C, mode of injection splitless, and carrier gas flow 1.5 mL min⁻¹. The run time was 29.67 min. Solvent delay was 4.00 min, temperature of the interface 295 °C, the acquisition mode was full scan and the scanning rate was 40.00 *m/z* to 550.00 *m/z*, MS source temperature 230 °C, and the MS quadrupole temperature 150 °C. "Wiley275", "NIST02", and "PMW_TOX3" MS libraries were used.

RESULTS AND DISCUSSION

Mass spectra of TCP and TAMORF

Figure 3 shows the mass spectra of pure TCP and TAMORF powders.

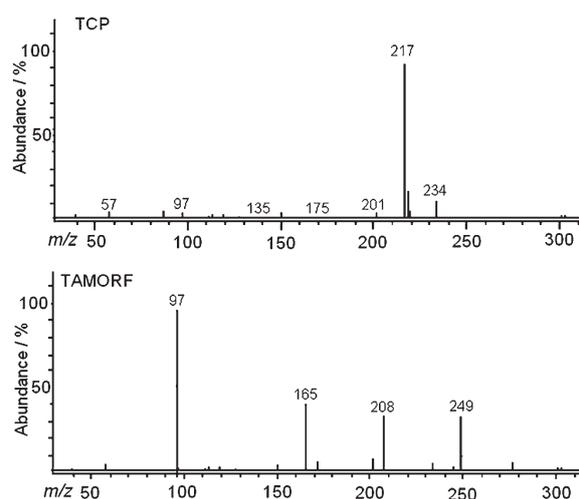


Figure 3 Mass spectra of TCP and TAMORF

GC-MS chromatograms of standard chloroform solutions of TCP and TAMORF

Total ion current (TIC) chromatogram of the standard chloroform solution of TCP showed two signals (Figure 4). The analysis of the mass spectra

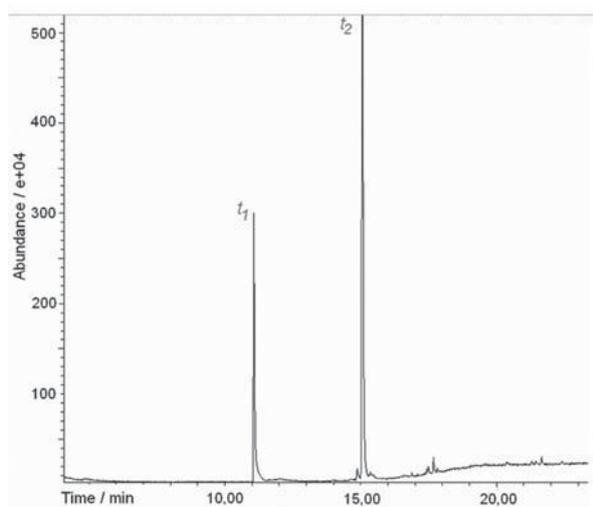


Figure 4 GC-MS chromatogram of TCP dissolved in chloroform

of signals at retention times t_1 and t_2 , and by their comparison with the mass spectra database showed that the signal t_1 corresponded to 1-thienylcyclohexene (Figure 5), and the signal t_2 to TCP (Figure 3). In other words, a part of TCP dissolved in chloroform remained unchanged and a part decomposed to 1-thienylcyclohexene. Structurally, thienylcyclohexene is TCP without the piperidine ring.

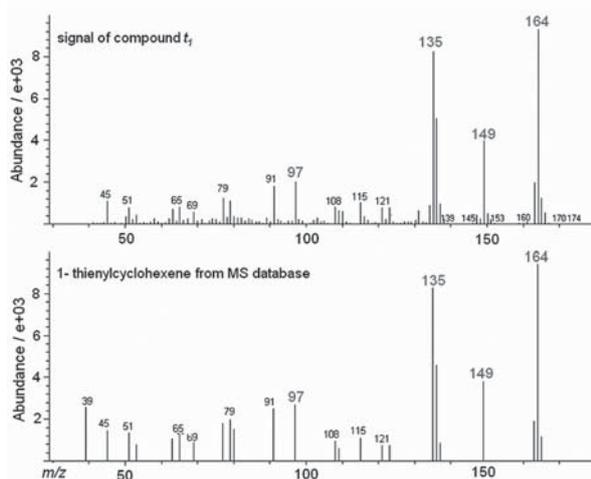


Figure 5 Mass spectrum of compound t_1 from Figure 4 and mass spectrum of thienylcyclohexene from mass spectra database

The TIC chromatogram of TAMORF standard solution (Figure 6) showed that TAMORF dissolved in chloroform decomposes to four compounds with signals at retention times t_1 - t_4 . Table 1 shows the five most abundant signals from the mass spectra of

compounds by retention times. No signal matches were found in the mass spectra database, which means that mass spectra of TAMORF and/or compounds to which it decomposes when dissolved in chloroform do not exist in common mass spectra databases.

Table 1 Signals from the mass spectra of compounds at retention times t_1 - t_4 from the chromatogram shown in Figure 6

Retention time	m/z
t_1	216, 161, 97, 175, 128
t_2	218, 97, 79, 123, 135
t_3	217, 97, 111, 79, 151
t_4	111, 234, 217, 201, 150

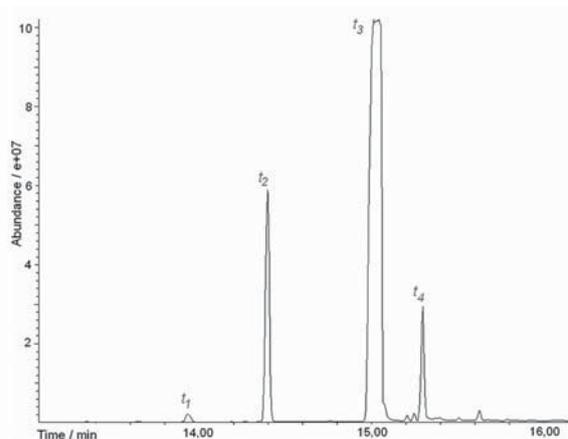


Figure 6 GC-MS chromatogram of TAMORF dissolved in chloroform

GC-MS chromatograms of TCP and TAMORF dissolved in urine

TIC chromatograms of TCP and TAMORF dissolved in urine showed signals at the same retention times as TIC chromatograms of these compounds dissolved in chloroform (Figures 4 and 6). The mass spectra of TCP and TAMORF dissolved in urine were equal to TCP and TAMORF dissolved in chloroform, which suggests that both TCP and TAMORF have the same decomposition pattern when dissolved in chloroform and urine.

TIC chromatograms of urine extract of rats treated with TCP and TAMORF

Figure 7 shows urine TIC chromatogram of urine extract of rats treated with TCP and the respective mass spectrum of a compound with signal at retention time t_1 . This mass spectrum corresponds to 1-thienylcyclohexene (Figure 5). GC-MS analysis

shows that rat body metabolises TCP into the same compound into which it decomposes when dissolved in chloroform or urine.

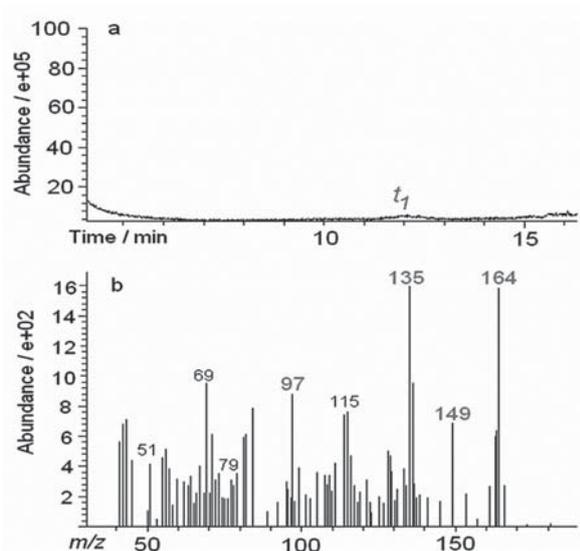


Figure 7 TIC chromatogram of urine extract of rats treated with TCP (a) and mass spectrum of a compound t_1 (b)

Figure 8 shows TIC chromatograms of urine extract of rats treated with TAMORF and controls (treated with glycerol formal and saline). The chromatogram of urine extract of rats treated with TAMORF (A) differs from the other two chromatograms (B and C) in ten signals. Rats treated with glycerol formal (B) showed signals at the same retention times t_1 - t_4 as rats treated with TAMORF (A), but their mass spectra were different. This means that signals t_1 - t_4 of the chromatograms shown in Figure 8A and 8B correspond to different compounds and suggests that signals t_1 - t_{10} from the chromatogram in Figure 8A originate from TAMORF excreted in rat urine. To verify that, we compared the chromatogram in Figure 8A with the chromatogram of TAMORF dissolved in chloroform or in urine (Figure 6). Both chromatograms have signals at retention times t_1 - t_4 and they correspond to the same mass spectra (see Table 1). Furthermore, TIC chromatogram of urine extract of rats treated with TAMORF also has signals at retention times t_5 - t_{10} (Figure 8A), which correspond to the mass spectra signals in Table 2.

Again we found no matches between the mass spectra of compounds with signals at retention times t_5 - t_{10} and the mass spectra databases.

Signals from the chromatogram in Figure 8A at retention times t_1 - t_4 originate from TAMORF which passed unchanged through rat body and decomposed

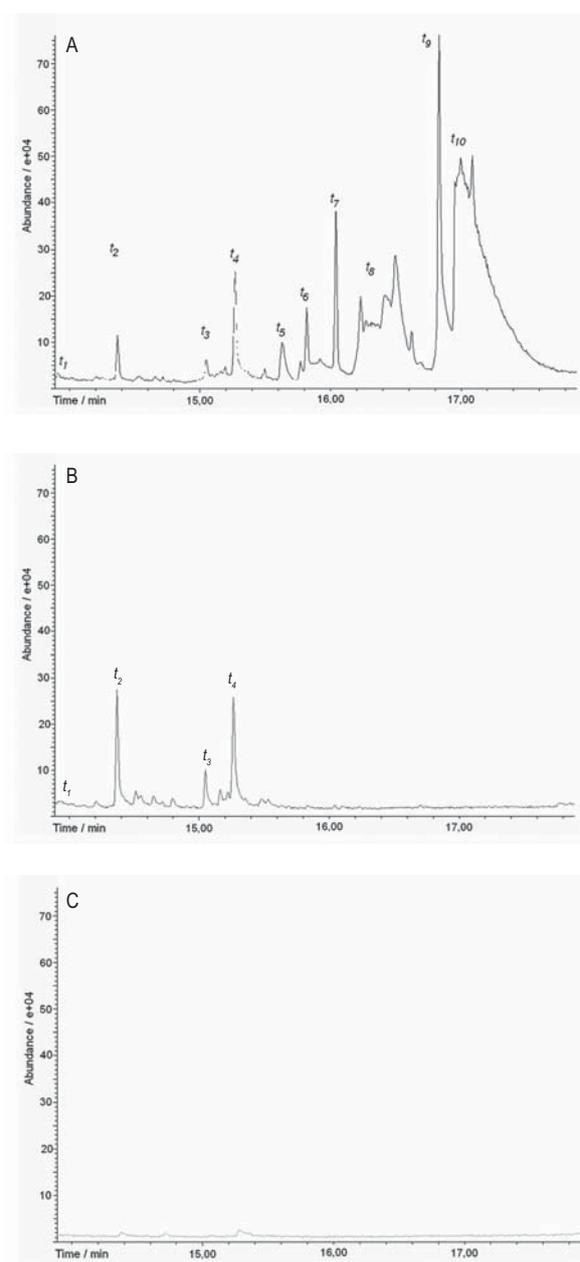


Figure 8 TIC chromatograms of urine extract of rats treated with TAMORF (A), glycerol formal (B), and saline (C)

Table 2 Signals from the mass spectra of compounds at retention times t_5 - t_{10} from the chromatogram shown in Figure 8A

Retention time	m/z
t_5, t_8	234, 97, 83, 123, 281
t_6	162, 204, 232, 97, 135
t_7	232, 281, 97, 125, 135
t_9	233, 111, 161, 248, 55
t_{10}	111, 233, 250, 217, 55

in rat urine the same way it did when it was dissolved in chloroform or in urine (Figure 6). In contrast, the chromatogram in Figure 8A shows signals at retention times t_5 - t_{10} , which do not correspond to any standard TAMORF solution signal. This suggests that a part of TAMORF was metabolised in the rats' body.

CONCLUSION

Our newly developed GC-MS method has been successful in establishing TCP and TAMORF decomposition in chloroform and rat urine, and TCP elimination via rat urine.

TCP dissolves well in water, chloroform, and rat urine. When dissolved, part of it remains unchanged, and part decomposes to 1-thienylcyclohexene. In our experiment all of TCP was metabolised in rats six hours after treatment. Further research should reveal how long rat body takes to completely decompose TCP, that is, for how long TCP remains active in rats.

TAMORF dissolves well in chloroform and glycerol formal and decomposes to four compounds, whose structure is yet to be determined by future studies. In rat urine, we found six TAMORF metabolites (also to be identified in future studies), which leads to the conclusion that six hours after administration, TAMORF can still be active.

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Sažetak

KVALITATIVNA PROCJENA ELIMINACIJE TCP-A I TAMORF-A IZ ORGANIZMA ŠTAKORA METODOM GC-MS

Živčani bojni otrovi po strukturi su organofosforni (OP) spojevi, čija je zajednička značajka ireverzibilna inhibicija acetilkolinesteraze (AChE), enzima koji hidrolizira acetilkolin (ACh) u živčanom sustavu. Patofiziološka zbivanja koja nastaju pri otrovanju OP-spojevima primarno su posljedica akumuliranog ACh na kolinergičkim receptorima i u središnjem živčanom sustavu. Još uvijek nesavršen, standardni tretman liječenja otrovanja OP-spojevima uključuje kombiniranu primjenu estera karbamata, atropina, oksima i antikonvulziva. Kako bi se unaprijedila uobičajena terapija, osobito kod otrovanja somanom, ispituju se antidotski učinci mnogih spojeva. Tenociklidin (TCP) i njegov adamantanski derivat TAMORF pokazali su zanimljiva svojstva pomoćne terapije pri otrovanju somanom. Kako bi se proširile dosadašnje spoznaje o načinu na koji tenociklidini štite organizam od trovanja OP-spojevima te također o trajanju njihova antidotskog učinka, u ovom radu razvijena je GC-MS-metoda za praćenje eliminacije TCP-a i TAMORF-a iz organizma. Rezultati GC-MS-analize pokazali su da šest sati nakon tretiranja štakora TCP-om mokraće sadržavaju metabolit TCP-a 1-tienilcikloheksen, dok šest sati nakon tretiranja štakora TAMORF-om mokraće sadržavaju i TAMORF i njegove metabolite. Drugim riječima, šest sati nakon tretmana TCP se potpuno metabolizira, dok se TAMORF metabolizira djelomično, a djelomično ostaje nepromijenjen.

KLJUČNE RIJEČI: *antidot, kvalitativna metoda, metabolit, mokraća, tenociklidin*

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