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Determination of the trypanocidal drugs Homidium, Isometamidium and Quinapyramine in bovine serum or plasma using HPLC

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Summary

Sensitive HPLC analytical methods for trypanocidal drugs in serum or plasma have been developed. The methods are suitable for the analysis of drugs that are widely used against animal trypanosomiasis at present, including Homidium bromide (Ethidium, Boots Company Ltd.), Homidium chloride (Novidium, May and Baker Ltd.), Isometamidium chloride (Samorin, May and Baker Ltd.), Quinapyramine chloride (Antrycide, ICI) and Quinapyramine sulphate (Trypacide, May and Baker Ltd.). The detection limits for various drugs range between 3 and 20 ng per ml of serum, when serum volumes up to 10 ml have been processed. These methods provide a significant improvement of sensitivity for Isometamidium over existing analytical procedures and represent new methods for analysis of Homidium and Quinapyramine.

Key words: trypanocidal drugs; Homidium; Isometamidium; Quinapyramine; serum; HPLC.

Introduction

Trypanosomiasis is responsible for preventing or severely restricting the utilization of about 7 million km² of land in Africa capable of supporting livestock. Vaccines are not available against the disease, thus the curative and preventive use of trypanocidal drugs is the most prevalent method for the control of trypanosomiasis in the estimated 50 million cattle within the tsetse belt. Drug

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therapy at present relies almost entirely on 4 compounds: Homidium, Diminazene Aceturate (Berenil, Hoechst AG), Isometamidium and Quinapyramine to which may be added Suramin (Naganol, Bayer Leverkusen) as a drug used to treat infections of *Trypanosoma evansi* in camels.

In its report of 1979 the Joint WHO/FAO Committee, recognizing that approximately 25 million doses of trypanocidal drugs were being administered annually, and that the costs of developing new drugs were becoming prohibitive, recommended that efforts be made to examine in detail the efficacy, mode of action and residual levels of existing drugs. Therefore the use of these drugs would be optimized for the control of trypanosomiasis and potential drug residues in meat and milk minimized. The development of a sensitive analytical technique described in this paper will be a first step to meet these demands.

Among the commonly used trypanocides there exist only published residue analysis procedures for Isometamidium and Berenil (Raether et al., 1972).

Isometamidium was analyzed after extraction from serum by Philips et al. (1967). A similar method was used by Braide and Eghianruwa (1980) who extracted Isometamidium from strongly alkaline medium with diethyl ether, followed by re-extraction into acetate buffer (pH 4.7) and measurement of the absorbance in a spectro-photometer at 378 nm. Both papers report a detection limit of about 1 μg per ml of serum.

Experimental materials and Instrumentation

Ethidium was obtained from Boots Company Ltd (Nottingham, England), Samorin from May and Baker Ltd. (Dagenham, England), and Antrycide from the German Technical Cooperation Agency (GTZ Chemotryp Project Nairobi, Kenya). Acetonitrile for chromatography was obtained from Merck (Federal German Republic). The disposable silica gel extraction columns (Catalog Nr. 7086-3) were manufactured by Baker Chemical Co. (Phillipsburg, N.J., USA).

HPLC was performed on a Waters Associates instrument with a Model M-45 solvent delivery system, fitted with a model U 6K injector, a Lambda Max model 480 UV detector, a Shimadzu Fluorescence HPLC Monitor model RF-530 and a Data module model M 730. The column was μ-Bondapak-C18 (0.45 × 30 cm) reversed phase, 10 μ particle size, supplied from Waters Associates. Degassed $CH_3CN/H_2O/HClO_4$ (40/60/0.03 v/v) and (20/80/0.03 v/v) solvents were used. The addition of 0.03% of 60% $HClO_4$ to the mobile phase proved necessary for eluting the drugs from the analytical column. The UV-spectra were recorded on a Lambda Max model 480 variable wavelength detector.

Methods

The trypanocides were added to defibrinated bovine blood or serum. If fresh blood is drawn from trypanocide treated animals, we recommend the addition of either 1 mg of the anticlotting agent disodium EDTA to 1 ml of blood or 0.15 ml of an anticlotting solution described by Rapatz and Luyet (1968) (2.2 g sodium citrate, 0.8 g citric acid and 2.45 g glucose in 100 ml water) to 1 ml blood. Both anticoagulants have proved not to interfere with the analy-

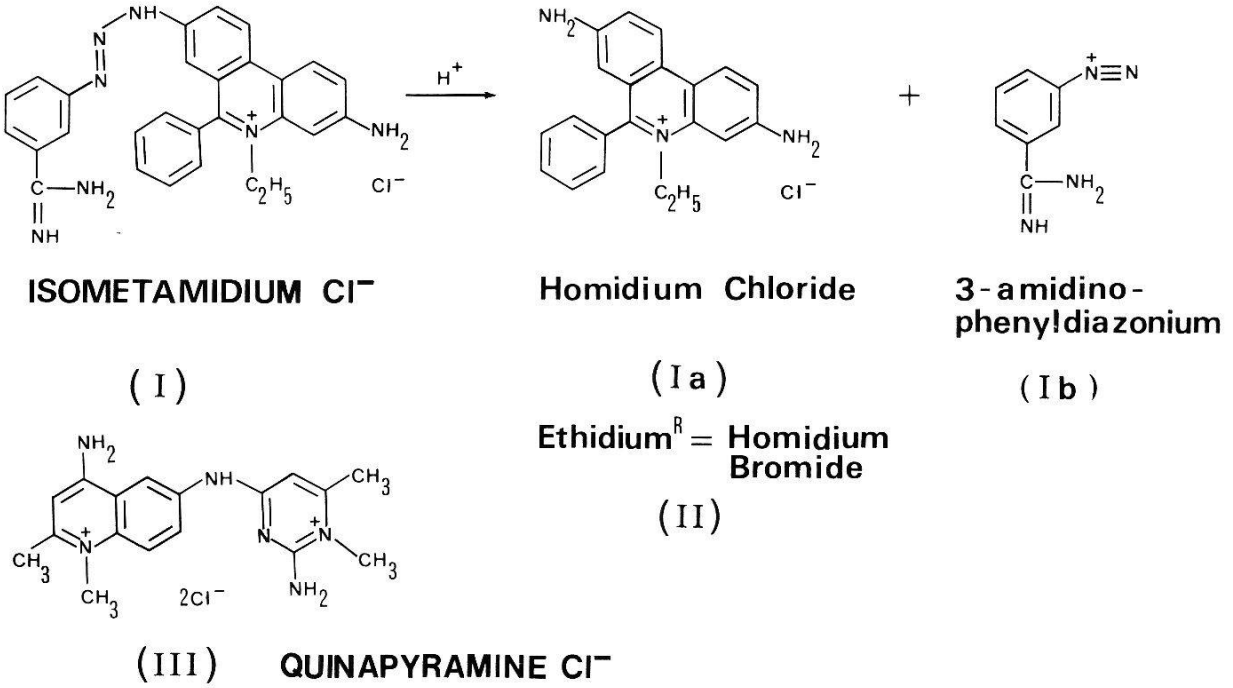

Fig. 1. Chemical structures of the trypanocides and cleavage of Isometamidium.

sis of the drugs in the resulting plasma. Therefore, either serum or plasma can be utilized in the analysis.

The preconcentration of Homidium, Isometamidium and Quinapyramine on silica gel prior to the determination by HPLC was a straight forward process, because each has functional groups that result in the compound being retained on a silica gel column. The drugs can then be eluted with acidified organic solvent-water mixtures. The clean up on the silica gel columns excluded any interfering serum or plasma components which could co-elute with the drugs from the analytical column. This had been verified with controls of serum samples processed without addition of drugs. The use of plastic vial instead of glass was necessary to avoid adsorption of the drug to the glass. The collection and storage of the samples in polyethylene vials created no problems as far as contamination of the samples with plasticizers is concerned. Homidium and Quinapyramine are stable under these eluting conditions, but the diazoamino group of Isometamidium is unstable at acidic pH. Isometamidium (I) cleaves to 3-amidinophenyldiazonium chloride (Ib) and Homidium (Ia) (Fig. 1). The fragments could be identified by their absorption spectra as well as by their retention times on the analytical column, using the corresponding reference compounds. The aromatic amidinophenyldiazonium chloride is quite stable at room temperature due to mesomerism and reacts only very slowly with water to form the corresponding phenol derivative. On the other hand, the 3-amidinophenyldiazonium chloride couples in light conditions with the other cleavage fragment to form azocompounds. This could be shown in the case of Isometamidium, where diazonium reacts with the Homidium to yield an azocompound

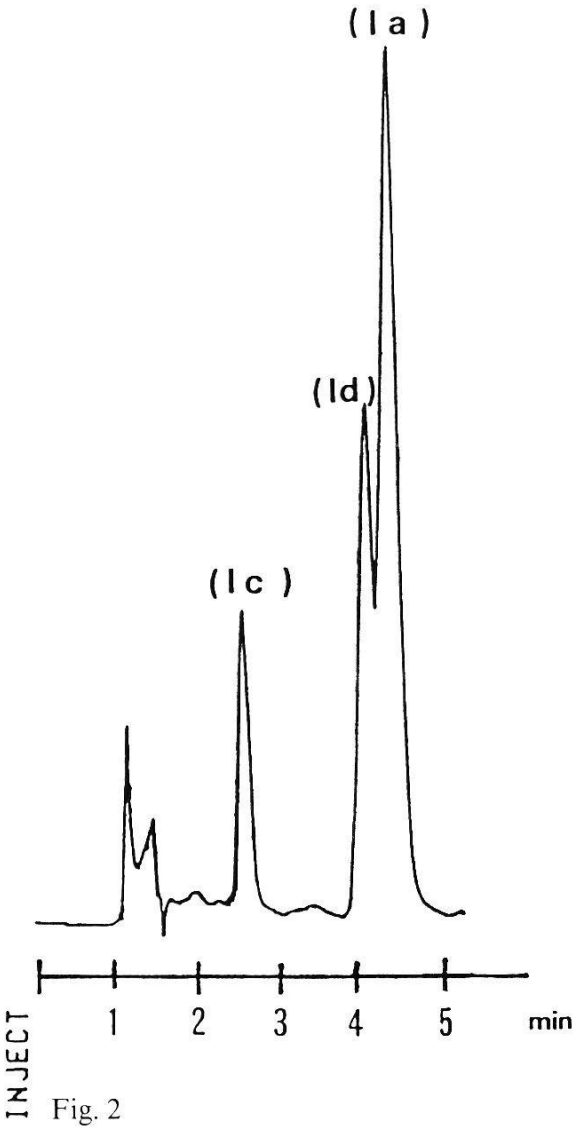

Fig. 2

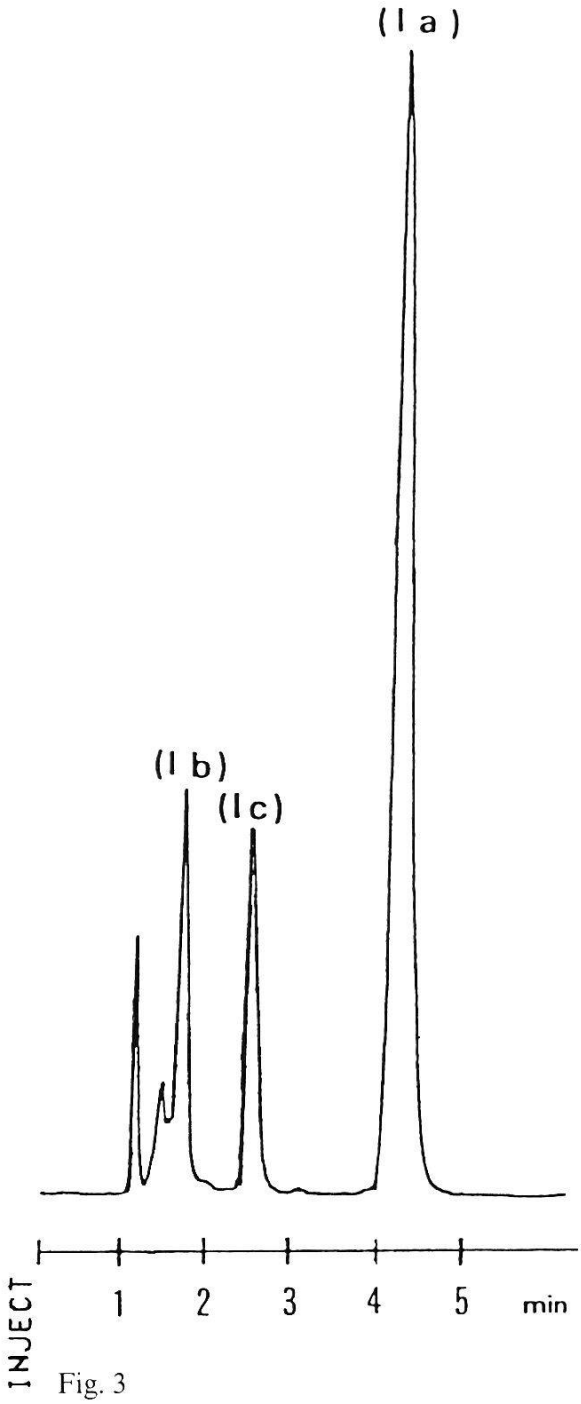

Fig. 3

Fig. 2. Isometamidium cleavage unter light conditions. Mobile phase $CH_3CN/H_2O/HClO_4$ (40/60/0.03 v/v). Solvent flow 2 ml/min, detection at 292 nm. Ia) Homidium; Ic) unknown impurity from Isometamidium; Id) coupling reaction product.

Fig. 3. Isometamidium cleavage under exclusion of light. Mobile phase $CH_3CN/H_2O/HClO_4$ (40/60/0.03 v/v). Solvent flow 2 ml/min, detection at 292 nm. Ia) Homidium; Ib) 3-amidinophenyldiazonium; Ic) unknown impurity from Isometamidium.

(Id) (Fig. 2). This reaction must be avoided to prevent losses of Homidium, which serves for the determination of Isometamidium. Therefore, during elution from the preconcentration column and storage of the eluate light must be excluded (Fig. 3). This was achieved by protecting the preconcentration column and the polyethylene vessel from light by wrapping with black adhesive tape.

Table 1. Analytical conditions for the clean up and determination of the trypanocidal drugs in bovine serum

Drug	Elution from the columns		UV and fluorescence	
	Preconcentration	Analytical	Compound	nm
Homidium	$CH_3CN/H_2O/HClO_4$	$CH_3CN/H_2O/HClO_4$	Homidium	292
Isometamidium[1]	40/59/1	40/60/0.03		
Quinapyramine	dito	$CH_3CN/H_2O/HClO_4$ 20/80/0.03	Quina-pyramine	emission 405

[1] After elution from the silica gel preconcentration column, Isometamidium spontaneously degrades to Homidium and 3-amidinophenyldiazonium chloride. Quantitation was affected by the quantitative analysis of the Homidium component.

From 1 to 10 ml serum are passed through the disposable silica gel plastic column (8 mm i.d., filling height 20 mm, average particle diameter 40 μm) previously washed with methanol and equilibrated with distilled water, by aid of a syringe connected to the top of the column. The drugs can be eluted from the column with 3 ml $CH_3CN/H_2O/HClO_4$ (40/59/1 v/v) after rinsing it with 3 ml distilled water, followed by 3 ml ethanol to remove impurities and finally washing it with another 3 ml water.

Isometamidium can be eluted quantitatively from the silica gel column with 3 ml $CH_3CN/H_2O/HClO_4$ (40/59/1 v/v). The eluate is collected into a polyethylene vial and stored for one hour in the dark for complete cleavage. An aliquot of the eluate is then injected onto the analytical column using $CH_3CN/H_2O/HClO_4$ (40/60/0.03 v/v) as the mobile phase at a flow rate of 2 ml/min. The Homidium peak serves for the determination (Table 1) and is recorded at the absorption maximum of 292 nm. For concentrations of 0.02–2.0 μg/ml serum, 93.3–99.4% of the Isometamidium was recovered when volumes representing up to 5 ml serum were processed on the preconcentration column. Sample volumes from 100–1000 μl were injected (Table 2). Fig. 3 shows the complex chromatogram of Isometamidium (II) with the cleavage products Ib and homidium (Ia) recorded at the absorption maximum for Homidium (292 nm). Compound Ic in the chromatogram is an unknown impurity of Isometamidium. The light catalyzed coupling of the two cleavage products of Isometamidium, namely the diazonium ion and Homidium is shown in Fig. 2. While compound Ib has disappeared a new compound Id is observed as a peak ahead of the simultaneously decreasing peak of Homidium (Ia). To convert all the Homidium to the azocompound will only be possible by adding an excess of the diazonium compound to the reaction mixture, because all available diazonium had already reacted (see Fig. 2). First of all the diazonium compound is not available commercially and secondly the formation of the azo dye will not

Table 2. Recoveries of the drugs from serum samples

Serum (ml)	Homidium added to serum		Recovery (%) (X ± SD)	Sample volume injected (µl)
	ng	ppb		
10	50	5	99.4 ± 1.4	1000
5	50	10	99.4 ± 2.3	500–1000
5	100	20	99.7 ± 1.6	500
5	250	50	98.6 ± 2.7	250
3	300	100	97.8 ± 1.2	250
3	600	200	99.0 ± 0.4	100
2	1000	500	99.7 ± 0.3	100
1	1000	1000	100.0 ± 0.2	100
1	2000	2000	99.8 ± 0.3	50
Serum (ml)	Isometamidium added to serum		Recovery (%) (X ± SD)	Sample volume injected (µl)
	ng	ppb		
5	100	20	93.3 ± 1.6	1000
5	250	50	94.3 ± 0.9	500–1000
3	300	100	94.9 ± 1.1	500
3	600	200	94.7 ± 0.9	250
1	500	500	99.0 ± 1.5	250
1	1000	1000	98.3 ± 0.1	250
1	2000	2000	99.4 ± 0.9	100
Serum (ml)	Quinapyramine added to serum		Recovery (%) (X ± SD)	Sample volume injected (µl)
	ng	ppb		
10	30	3	99.0 ± 2.7	250
5	25	5	100.0 ± 2.8	250
5	50	10	98.2 ± 3.0	250
5	100	20	99.3 ± 2.7	250
2	100	50	99.6 ± 2.4	250
2	200	100	99.2 ± 1.6	250
2	400	200	99.5 ± 1.8	250
1	500	500	99.6 ± 1.4	250
1	1000	1000	98.0 ± 2.2	100
1	2000	2000	99.6 ± 0.6	50

be desirable because of the possible formation of isomers. To avoid this interference the Isometamidium cleavage products must be protected from light (compare Fig. 2 with Fig. 3).

Homidium and Quinapyramine are eluted from the silica gel column with the same solvent mixture as used with Isometamidium. Immediately after elu-

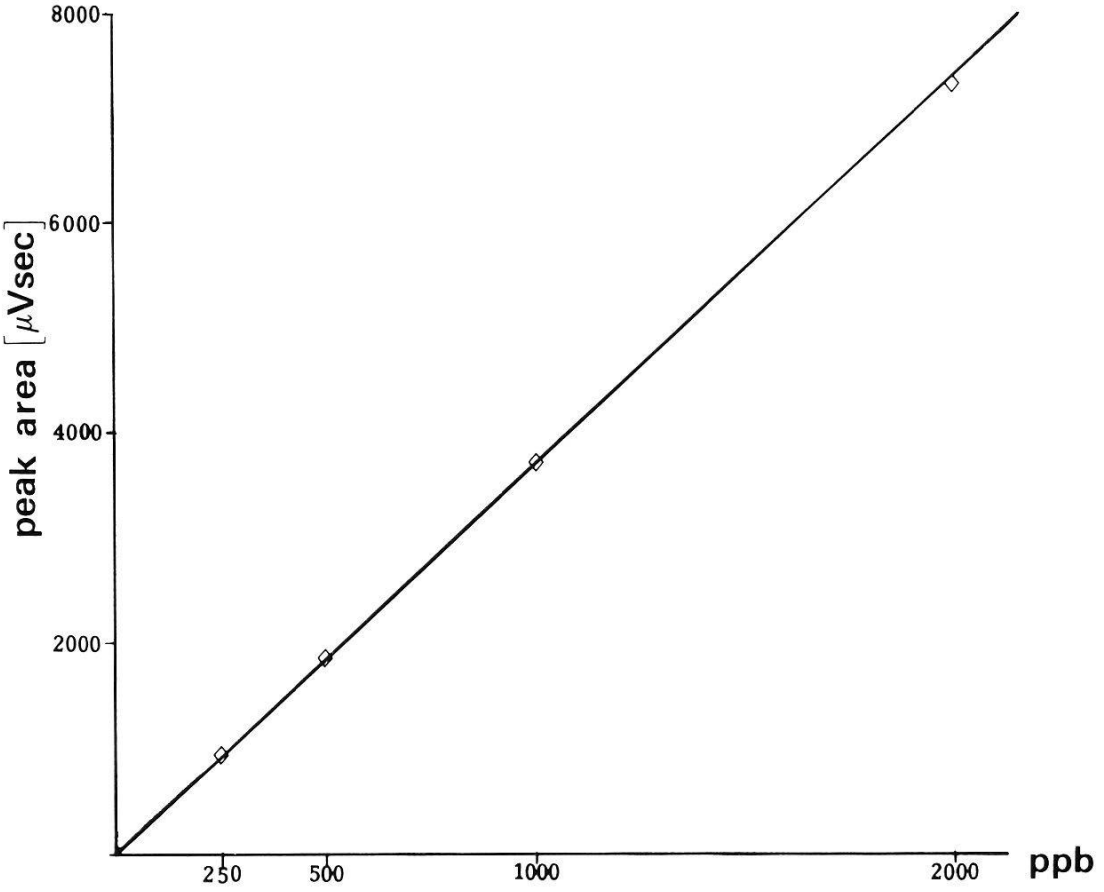

Fig. 4. Calibration curve for Homidium. Peak area versus concentration (ppb).

tion, an aliquot of the solutions can be injected onto the analytical column using the solvent system $CH_3CN/H_2O/HClO_4$ (40/60/0.03 v/v) for the chromatographic determination of the Homidium, detecting the peak at 292 nm. The recovery for Homidium in the concentration range of 0.005–2.0 μg/ml serum was between 97.8 and 100% and sample volumes from 50 to 1000 μl were injected (Table 2). The stable compound Homidium gives a single peak at the absorption maximum of 292 nm, molar absorption coefficient ε = 52.800 (retention time 4.52 min). For the determination of Quinapyramine, the solvent system $CH_3CN/H_2O/HClO_4$ (20/80/0.03 v/v) at a flow rate of 2 ml/min was used, detecting the peak at the emission wavelength of 405 nm (excitation at 296 nm, molar absorption coefficient ε = 35.500) on the fluorescence detector. The recovery for Quinapyramine in the range of 0.003–2.0 μg/ml serum was between 98.0 and 100% and sample volumes up to 250 μl were injected (Table 2). The stable compound Quinapyramine gives a single peak at the emission wavelength of 405 nm with a retention time of 3.32 min.

The recoveries of the drugs from serum are shown in Table 2. The values given are the means of three replications. The recoveries were determined by comparing the values obtained from serum samples, spiked with known amounts of the drugs with the calibration curve made each day by plotting 3 or

4 concentration values in the desired range. A standard solution of the drugs in water was prepared daily and known amounts of the aqueous solution were added to a solution of $CH_3CN/H_2O/HClO_4$ (30/69/1 v/v), from which aliquots were injected onto the analytical column. The Samorin calibration mixture has to be stored for one hour in the dark before injection. The standard calibration curves were linear over the entire concentration range given in Table 2. An example of a calibration curve for Homidium in the range from 250 to 2000 ppb is given in Fig. 4.

Conclusion

Analytical procedures for sensitive determination of the trypanocidal drugs Homidium, Isometamidium and Quinapyramine in bovine serum or plasma have been developed. The steps of the procedures are: separation of plasma from the blood cells by centrifugation, preconcentration on small silica gel columns, elution with an acidified solvent and HPLC measurement with UV or fluorescence detection.

All recoveries were between 93.3 and 100%. The effective detection limits processing 1 ml of serum were 50 ng for Homidium, 100 ng for Isometamidium and 30 ng for Quinapyramine. Processing larger volumes of up to 10 ml serum, the detection limits were 5 ng per ml serum for Homidium, 3 ng per ml serum for Quinapyramine and 20 ng per ml serum for Isometamidium.

The analysis of Samorin is based on acidic cleavage of Samorin to Homidium. This method is indirect and will not, of course, distinguish between Homidium and Samorin, if the former is also present in the sample whether metabolically formed from Samorin or resulting from previous treatments with Homidium.

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