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Note

Separation of hordenine and N-methyl derivatives from germinating barley by liquid chromatography with dual-electrode coulometric detection

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Hordenine [4-(2-dimethylaminoethyl)phenol] is chemically closely related to tyramine and several other compounds normally present in mammalian tissues and body fluids. Hordenine itself, however, has not been reported to be a normal constituent in mammals. Hordenine has earlier been used in both human and veterinary medicine in the treatment of diarrhoea and other intestinal disturbances[1,2].

Hordenine has been detected in the urine of race horses[3]. To date, the Medication Control Programme of Swedish horseracing has found four urine samples to contain hordenine, mainly in the form of an acid-labile conjugate, in the concentration range 1–43 μg/ml. The analysis was performed by thin-layer chromatography (TLC) and the presence of hordenine was confirmed by gas chromatography–mass spectrometry (GC–MS).

This work is part of a larger study in which various horse feeds on the Swedish market and plant materials were analysed for the presence of hordenine. Further, the pharmacokinetics of hordenine in the horse and the effects on cardiorespiratory and blood lactate responses to exercise in the horse were studied[4]. Feeding one horse 733 g of germinating barley, a natural source of hordenine containing 63 μg/g of hordenine, resulted in hordenine concentrations in the urine comparable to those mentioned above[4]. The hordenine content in the germinating barley was analysed both by GC–MS[4] and by the present liquid chromatographic (LC) method. Dried barley seedlings (from the malthouse industry) and parts of reed canary grass, also a natural source of hordenine, were analysed by LC with ultraviolet detection[4].

The present chromatographic study of hordenine also included the corresponding N-desmethyl derivatives, tyramine and N-methyltyramine. These are precursors in the formation of hordenine in barley seedlings and certain other plants, and have been reported to occur in dried barley seedlings from the malthouse industry, as also does N-trimethyltyramine (candicin)[5]. Pholedrine [4-(2-methylaminopropyl)phenol] was tested for comparison.

EXPERIMENTAL

Apparatus

The LC system consisted of a Constametric III pump (LDC, Riviera Beach,

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FL, U.S.A.), a Wisp 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.) and either a UV detector (Spectro Monitor III; LDC) operated at 273 nm or an electrochemical detector (Model 5100 A; ESA, Bedford, MA, U.S.A.). The electrochemical detection system consists of a coulometric guard cell (Model 5020) and a solid-state analytical cell containing dual coulometric working electrodes made from porous graphite (Model 5010). The guard cell, operated at +0.8 V, was connected between the pump and injector to reduce the background current at the analytical cell resulting from oxidation of contaminants in the eluent. For the detection of hordenine and related compounds, the first working electrode (D_1) was set at +0.5 V, a potential at which hordenine just begins to oxidize according the hydrodynamic voltammogram of hordenine obtained for the LC system with phosphate buffer (pH 3.0) –methanol (80:20, v/v) as the eluent. The electro-oxidation of the compounds was then effected at the working electrode (D_2) at +0.75 V. The signal from D_2 was recorded and integrated using a Chromatopac C-RIB integrator (Shimadzu, Kyoto, Japan).

The pH of the chromatographic eluent was recorded with a Metrohm (Herisau, Switzerland) Model 620 pH meter.

Chemicals and reagents

Hordenine hemisulphate was obtained from Sigma (St. Louis, MO, U.S.A.) and tyramine hydrochloride from Merck (Darmstadt, F.R.G.). N-Methyltyramine and N-trimethyltyramine iodide (candicin) were synthesized according to Gruenke *et al.*[6] and Buck *et al.*[7], respectively. The structures of the compounds are given in Fig. 1.

N,N-Dimethyl-N-octylamine (DMOA) was obtained from ICN Pharmaceuticals (Planview, NY, U.S.A.) and sodium octylsulphate from Merck. Tetrabutylammonium (TBA) hydroxide was prepared from tetrabutylammonium iodide (Merck)[8] and the titre was determined by the picric acid method[9].

The phosphate buffers were prepared from sodium dihydrogenphosphate and orthophosphoric acid. Methanol of analytical-reagent grade (Merck) was used.

Barley

First-class barley was soaked in water for 24 h and was then spread out on wet paper for 5 days.

To 1 g of germinating barley in a centrifuge tube were added 15 ml of 1 *M* hydrochloric acid and the tube was placed in a water-bath at 95°C for 30 min. The mixture was then homogenized (Ultra-Turrax; Janke and Kunkel, Staufen, F.R.G.) for 3 min and centrifuged. The supernatant was diluted 10 fold with the chromatographic eluent and 20 µl were injected onto the column.

Liquid chromatography

Initially the separation was made on a LiChroCART column system with a

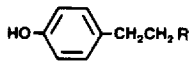

Fig. 1. Structures of the compounds. R = NH_2, tyramine; $NHCH_3$, N-methyltyramine; $N(CH_3)_2$, hordenine; $N^+(CH_3)_3$, N-trimethyltyramine.

LiChrosorb RP Select B cartridge (7-μm particle size; type 16365; Merck). The dimensions were 125 mm × 4.0 mm I.D. The eluent was a mixture of 0.1 *M* NaH_2PO_4 or 0.01 *M* NaH_2PO_4 (pH = 4.6) with methanol (90:10, v/v). In some experiments, DMOA (0.8 m*M*) and/or sodium octylsulphate (1–3 m*M*) were added. The flow-rate was 0.8 ml/min. The influence of DMOA in the eluent on the capacity factor and the peak shape on the LiChrosorb RP Select B column was compared with the results obtained with a 100 mm × 3.0 mm I.D. column packed with 10-μm Spherisorb ODS (Phase Separations, Queensferry, U.K.). To determine peak symmetry, the perpendicular from the peak maximum was drawn and the peak width at 13,5% of the height was used[10].

Later, a strong cation-exchange column, Nucleosil SA, 5-μm particle size (Macherey, Nagel & Co., Düren, F.R.G.) was used. The dimensions of the column were 200 mm × 4.6 mm I.D. The eluent was phosphate buffer (pH 3.0, ionic strength = 0.1)–methanol (80:20, v/v) at a flow-rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Addition of dimethyloctylamine (DMOA) to the eluent

The LiChrosorb RP Select B column is designed to give a good chromatographic performance with basic substances[11]. Nevertheless, the peaks obtained for hordenine and its N-methyl derivatives tailed significantly, giving A_s = 2.3–3.3 with 0.1 *M* NaH_2PO_4 as the eluent. An improvement in peak symmetry and the influence on retention on reversed-phase columns by DMOA in the eluent have been demon-

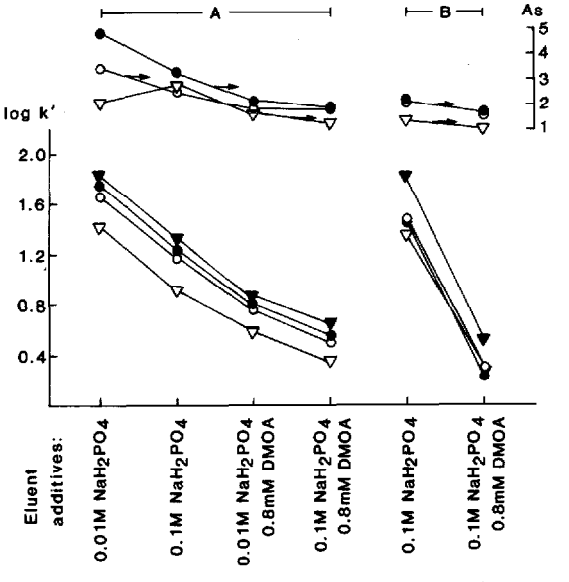

Fig. 2. Comparison of the capacity factors and peak symmetry. Column (A) LiChroCART with LiChrosorb RP Select B; (B) Spherisorb ODS. Eluents: mixture of 0.1 *M* NaH_2PO_4 or 0.01 *M* NaH_2PO_4 (pH = 4.6) with methanol (90:10 v/v), containing 2 m*M* octylsulphate and with 0.8 m*M* DMOA added to three of the systems as indicated. Sample: ▽ = tyramine; ▼ = pholedrine; ○ = hordenine; ● = N-trimethyltyramine.

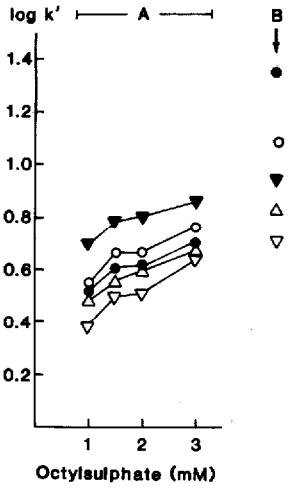

Fig. 3. Comparison of capacity factors. (A) Column: LiChroCART with LiChrosorb RP Select B. Eluent: 10% methanol in 0.01 *M* NaH_2PO_4 (pH = 4.6) containing DMOA (0.8 m*M*) and octylsulphate. (B) Column: Nucleosil SA. Eluent: 20% methanol in phosphate buffer (pH 3.0, ionic strength = 0.1). Sample: ▽ = tyramine; △ = N-methyltyramine; ▼ = pholedrine; ○ = hordenine; ● = N-trimethyltyramine.

strated for more hydrophobic amines[12–16]. Addition of 0.8 m*M* DMOA to the eluent in this study improved the peak symmetry (*As* = 1.3–2.3). However, the retention of all substances decreased and the capacity factor for hordenine decreased from 14.6 to 3.3. The decrease in retention was even greater on the Spherisorb ODS column. Small changes in selectivity were obtained with both columns on addition of DMOA. The results are summarized in Fig. 2. The improvement in peak symmetry was most pronounced for candicin. A similar marked effect of additives in chromatographic solvents on other quaternary ammonium compounds has been reported[17].

However, the resulting retention times were too short to resolve the compounds of interest fully. Addition of octylsulphate to the eluent at concentrations up to 3 m*M* increased the retention (Fig. 3) but the separation was still not adequate. A change of the eluent pH to 3, a reduction in the ionic strength to 0.01 or exchange of methanol for acetonitrile did not improve the separation on the LiChrosorb RP Select B column.

Data from separations on the Spherisorb ODS column showed that the addition of a methyl group to the α-carbon of N-methyltyramine (giving pholedrine) gives an increase in log *k'* of 0.4 units. Methyl substitution at the nitrogen (giving hordenine) increases log *k'* by only 0.05 units. This ODS column seems to give a poor selectivity for different substituents at the nitrogen atom.

Different selectivities can often be observed with various columns of the same type, as for non-steroidal anti-inflammatory drugs on C_{18} columns (Spherisorb ODS *vs.* LiChrosorb RP-18)[18]. This is obviously due to different degrees of end-capping and other dissimilarities in manufacture. Some other brand of C_{18} material, not investigated by us, might possibly separate the compounds studied here. However, our present data suggest that a sufficient separation on this type of column cannot be

obtained, even if the number of theoretical plates could be reasonably increased, *e.g.*, by the use of longer columns and/or smaller particle size. Reversed-phase columns have been used previously to determine tyramine[19–21].

A considerable improvement in the separation was obtained with the Nucleosil SA column (Fig. 3), and this column was exclusively used subsequently in the study. The peak symmetries on the Nucleosil SA correspond to those on the Spherisorb ODS column (A_s = 1.5–2.2) without the addition of DMOA to the eluent. Catecholamines have also been separated on this type of column, which were shown to give cleaner chromatograms in the analysis of plasma and urine samples compared with LiChrosorb RP-18 columns[22,23].

Electrochemical detection

Hordenine showed an electrochemical response with the ESA coulometric detector and required a relatively high oxidation potential of +0.75 V. The same potential has been used for the coulometric detection of octopamine and tyramine[24]. Use of the ESA coulometric detector required a buffer system that generated low currents at both working electrodes. Hydrodynamic voltammograms recorded between 0.0 and +1.3 V showed that the background current was increased considerably by the addition of DMOA (Fig. 4). In this experiment, 24% acetonitrile in phosphate buffer (pH 3.25; ionic strength = 0.01) was used. In contrast, TBA did not increase the noise and could be used as an additive to the eluent for electrochemical detection at the high potential necessary to detect hordenine and related compounds. The separation of a standard mixture of the compounds on the Nucleosil SA column at a potential of +0.75 V is shown in Fig. 5. The calibration graph for hordenine was linear in the range 0.56–28 μM (0.092–4.6 μg/ml). The regression equation for the curve was $y = 38.5\,x - 24.2$ (r = 0.993; n = 8) and the detection limit was 0.33 μM (0.054 μg/ml) measured at three times the background noise.

In our earlier investigations[4], hordenine was determined by high-performance liquid chromatography with ultraviolet detection. It has a fairly strong absorbance at 275 nm (molar absorptivity ε = 1606 at pH 3.15), but the present method gave a 25-fold improvement in the detection limit (1.35 *vs.* 0.054 μg/ml).

Analysis of germinating barley and horse urine

A chromatogram of an extract of germinating barley is shown in Fig. 6. Peaks with the same retention times as tyramine, N-methyltyramine and hordenine were

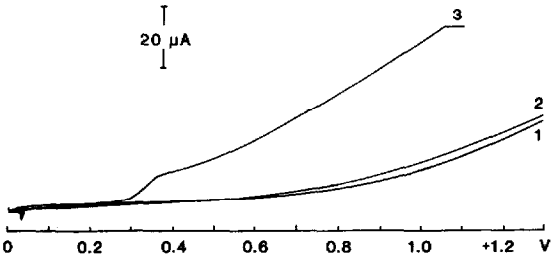

Fig. 4. Hydrodynamic voltammograms with different additives in the eluent. Eluent: 24% acetonitrile in phosphate buffer (pH 3.25, ionic strength = 0.01). 1 = eluent; 2 = elutent + TBA (0.8 mM); 3 = eluent + DMOA (0.8 mM).

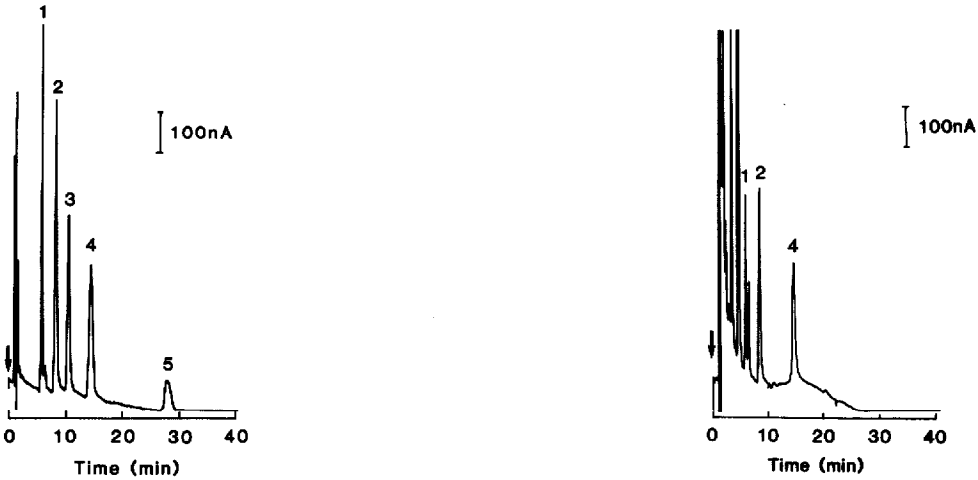

Fig. 5. Chromatogram of 10–19 ng of standards with D_1 1 at +0.5 V and D_2 at +0.75 V. Flow-rate: 1.0 ml/min. Column: Nucleosil SA. Eluent: 20% methanol in phosphate buffer (pH 3.0, ionic strength = 0.1). Peaks: 1 = tyramine (119 pmol); 2 = N-methyltyramine (129 pmol); 3 = pholedrine (93 pmol); 4 = hordenine (114 pmol); 5 = N-trimethyltyramine (67 pmol).

Fig. 6. Chromatogram of extract from germinating barley. Chromatographic conditions and peaks as in Fig. 5.

obtained. Determination of the concentrations from peak areas compared with standards analysed under the same conditions indicated that 1 g of barley contained 26 μg of tyramine, 48 μg of N-methyltyramine and 63 μg of hordenine. The presence of hordenine was confirmed by GC–MS[4].

The relative standard deviation for hordenine added to 1 *M* hydrochloric acid and treated as described under Experimental for barley extraction was 8.8% at the 26 μ*M* (4.3 μg/ml) level (n = 8). The yield through the sample work-up was 105%.

Samples of horse urine were diluted 10-fold with chromatographic eluent (without treatment with hydrochloric acid) and 20-μl aliquots were injected. However, this procedure proved unsuccesful for the determination of hordenine in the urine of horses that had received this compound. Unfortunately, horse urine contains large amounts of phenols and other electroactive compounds that seriously interfere with the determination of hordenine using electrochemical detection, and contamination of the electrode surfaces necessitates extensive cell cleaning every 4–6 injections.

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