Pyrrole detection and the pathologic progression of *Cynoglossum officinale* (houndstongue) poisoning in horses

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**Abstract.** Houndstongue (*Cynoglossum officinale*), a noxious weed that contains pyrrolizidine alkaloids (PAS), infests pastures and fields in the western United States and Europe. The purpose of this study was to develop techniques to better diagnose PA poisoning and describe the progression of gross and microscopic lesions caused by houndstongue intoxication. Six horses were gavaged daily with a suspension of houndstongue containing 5 or 15 mg/kg total PA for 14 days. Two horses were treated similarly with ground alfalfa as controls. Liver biopsy samples and serum biochemical and hematologic values were evaluated biweekly. Within 7 days after dosing, horses treated with 15 mg/kg PA developed severe liver disease characterized by altered bile acid metabolism, elevated serum enzymes, and extensive hepatocellular necrosis with minimal periportal fibrosis and biliary hyperplasia. The condition of these animals continued to deteriorate, and they were euthanized. For several weeks after dosing, horses treated with 5 mg/kg PA were depressed, had transient elevations of serum enzymes and bile acids, and developed minimal periportal hepatocellular necrosis with fibrosis. The biochemical changes resolved by 6-8 weeks; however, the histologic disease persisted with extensive megalocytosis by week 14. Throughout the study, the rate of hepatocellular proliferation remained constant. Biliary cells had an increase in mitotic rate that correlated with the histologic changes. Hepatic tissue-bound pyroroles (PA metabolites) were identified in necropsy samples of treated animals using gas chromatography/mass spectrometry and photometrically with Ehrlich’s reagent. These findings suggest that pyrrole extraction and identification are useful in documenting PA exposure and that houndstongue is extremely toxic to horses.

Houndstongue (*Cynoglossum officinale*), a weed of the plant family Boraginaceae, infests many pastures and fields in Europe and the USA. It is a biennial that grows 0.3-1.2 m tall in pastures, disturbed areas, and along roadsides (Fig. 1). It forms a rosette during the first year of growth and a flowering stalk in the second year. The leaves are rough, alternate, and 2-30 cm in length and are said to resemble a dog’s tongue. The flowers are reddish purple, and the fruit divides into 4 spine-covered 8-10-mm nutlets that cling to clothing or animals. Houndstongue is generally not palatable to livestock; however, when included in pelleted or stored forages, it is readily eaten. Livestock poisonings have been reported in the USA, Europe, and Russia.

Plants such as houndstongue that contain pyrrolizidine alkaloids (PAS) are found throughout the world, and human and livestock poisoning is frequently reported. Animal intoxication often manifests as a chronic loss of condition. Affected animals either die of hepatic failure or are culled as “poor doers.” Human poisonings occur when PA-containing plants are used medicinally or when they contaminate cereal crops.

Pyrrolizidine alkaloids are metabolized into toxic pyroroles by the mono-oxygenase system of the liver. The pyroroles quickly react and form adducts with hepatic proteins and nucleic acids. Hepatic changes associated with PA poisoning vary from fulminant necrosis to chronic hepatic fibrosis. Some animals develop abnormally large hepatocytes (megalocytes). Many of these histologic lesions are nonspecific and can be initiated by other toxic, infectious, and immune diseases.

A definitive diagnosis of houndstongue intoxication is often difficult to obtain. Most livestock poisonings occur from ingestion of contaminated hay or feed. Because exposed animals commonly develop signs of hepatic failure several weeks or months later, exact feeding history and feed samples often are no longer available. The purposes of this study were to better describe the sequential clinicopathologic alterations of houndstongue intoxication in horses, to determine the relative toxicity of houndstongue in horses, and to develop analytical techniques to identify PA residues in plant and animal tissues for documenting exposure.

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Materials and Methods

Experimental model. Houndstongue was collected near Logan, Utah, while in the flower stage. The plant was air dried, ground to pass through a 4-mm screen, and stored in plastic bags until feeding. The ground plant was analyzed, and the total PA concentration (free base and N-oxide) was determined by nuclear magnetic resonance spectroscopy using previously described methods.\textsuperscript{17,20} A short summary and abstract of the animal exposure and response has been previously published.\textsuperscript{22} Six 1-yr-old horses were gavaged daily via a nasogastric tube with ground houndstongue in 3 liters of warm water for 14 days. The high-dose group (3 animals) received a total PA dosage of 15 mg/kg/day, and the low-dose group received 5 mg/kg/day. Two control animals were gavaged with similar volumes of ground alfalfa during the same period. Serum, plasma, whole blood, and liver biopsies were collected and bromosulfophthalein (BSP) clearance assays were performed biweekly. The horses were euthanized following the onset of clinical illness. The remaining clinically normal animals were euthanized 252 days postexposure.

Pyrrole extraction and detection. Hepatic-bound PA metabolites (pyrroles) were detected using a modification of previously reported techniques.\textsuperscript{13,14,26} Five grams of liver were homogenized in 30 ml acetone and centrifuged at 1,500 x g for 15 min. The pellet was resuspended and centrifuged in 30 ml acetone and then in 30 ml absolute ethanol and was again resuspended and stirred in 30 ml ethanolic 3% silver nitrate with 0.2 ml trifluoracetic acid for 1 hr at 60 C. The digest was centrifuged at 1,500 x g for 15 min, and the supernatant was concentrated in a rotary vacuum evaporator. The residue was dissolved in 5 ml diethyl ether and mixed with 4 ml 10% aqueous potassium carbonate. The flask was rinsed with an additional 4 ml diethyl ether, and the combined sample was centrifuged. The ether layer was removed and placed on a silica column prepared in chloroform. The sample was eluted through the silica with 8 ml ether and concentrated by drying under nitrogen. The resulting sample was dissolved in 0.4 ml dry ethanol and analyzed either by direct detection using capillary gas chromatography/mass spectrometry (GC/MS) or by a modified Ehrlich’s reaction and spectrometry.\textsuperscript{13,15}

Clinical and histologic pathology. Hemograms, leukograms, and platelet counts were performed using an automated hematology analyzer.\textsuperscript{4} Serum biochemical values for
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Figure 2. Necrotizing dermatitis (photosensitivity) on the nose of a horse that was dosed with houndstongue containing 15 mg/kg total pyrrolizidine alkaloid for 14 days.

glucose (GLU), blood urea nitrogen (BUN), creatinine, phosphorus, calcium, albumin, total protein, albumin/globulin ratio, cholesterol, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatinine kinase (CK), sodium, potassium, sodium/potassium ratio, chloride, total carbon dioxide, anion gap, osmolality, Sorbitol dehydrogenase (SDH), and bile acids were determined using standard biochemical techniques. Coagulation parameters (activated partial thromboplastin time [PTT], prothrombin time, fibrinogen) were analyzed with an automated coagulation analyzer.

Hepatic biopsy samples and tissues collected at necropsy were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained for microscopic examination using standard techniques. Replicate sections were immunohistochemically stained for proliferating cell nuclear antigen (PCNA) using previously described techniques. Approximately 5,000 cells from each section of liver were counted, and the mitotic index for hepatocytes, biliary epithelium, and other cells (macrophages, endothelial cells, fibroblasts) was calculated. The area of hepatocyte nuclei in these sections was also measured using digital computer image analysis.

The hematologic results, serum biochemistry levels, cell proliferation assays, and morphometry were compared with a 95% confidence interval (± 2 SD) generated from the controls and pretreatment samples. Additionally, the means for each of the treatment groups were compared using an analysis of variance with a generalized linear model for a repeated measures design. Mean separations were done using Duncan’s method at P < 0.05 after a significant R test at a ≤ 0.5.

Results

Two to 3 days after the last dose, all 3 high-dose horses developed severe liver disease characterized by depression, anorexia, and icterus. One animal developed photosensitivity and necrotizing dermatitis (Fig. 2). The clinical condition of all 3 of these animals deteriorated until they were euthanized before week 5 of the study. The 3 low-dose animals were transiently depressed and lost weight for several weeks. Within 8 weeks, these clinical signs gradually resolved and all 3 horses gained weight with no clinical evidence of toxicosis through the end of the study.

Two of the high-dose horses developed a degenerative left shift with severe toxicity characterized by neutrophil granulation, vacuolated monocytes, and large vacuolated platelets. They also had a lymphopenia and eosinopenia. The hemogram of the high-dose horses and both the hemogram and leukogram of the low-dose horses were similar to those of the control animals. There were no significant changes (P > 0.05) in the mean thrombocyte count for any of the groups at any time; however, several of the high-dose horses
Table 1. Serum biochemical changes (x ± SD)* in horses dosed with pyrrolizidine alkaloid derived from *Cynoglossum officinale* (houndstongue) at 15 mg/kg/day (n = 3) and 5 mg/kg/day (n = 3).

<table>
<thead>
<tr>
<th>Analyte†</th>
<th>Normal range‡</th>
<th>High dose</th>
<th>Low dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 14</td>
<td>Day 28</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>65–125</td>
<td>81 ± 6</td>
<td>51 ± 39</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>11–31</td>
<td>14 ± 1</td>
<td>8 ± 1§</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>2.8–4.5</td>
<td>3.4 ± 0.4</td>
<td>2.7 ± 0.1§</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.2–2.8</td>
<td>4.5 ± 3.7</td>
<td>15.4 ± 4.9§</td>
</tr>
<tr>
<td>PT (sec)</td>
<td>25–43</td>
<td>57 ± 19</td>
<td>91 ± 35§</td>
</tr>
<tr>
<td>BSP (min)</td>
<td>1.8–3.8</td>
<td>7.9 ± 6.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Bile acids (µM/liter)</td>
<td>6.0–12.9</td>
<td>40 ± 52</td>
<td>68 ± 23§</td>
</tr>
<tr>
<td>ALT (U/liter)</td>
<td>117–309</td>
<td>213 ± 37</td>
<td>270 ± 17§</td>
</tr>
<tr>
<td>GGT (U/liter)</td>
<td>10–25</td>
<td>22 ± 10</td>
<td>364 ± 501</td>
</tr>
<tr>
<td>AST (U/liter)</td>
<td>72–336</td>
<td>418 ± 303</td>
<td>1,009 ± 836</td>
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<tr>
<td>SDH (U/liter)</td>
<td>23–69</td>
<td>162 ± 55§</td>
<td>914 ± 566§</td>
</tr>
<tr>
<td>LDH (U/liter)</td>
<td>108–340</td>
<td>280 ± 149</td>
<td>947 ± 881</td>
</tr>
<tr>
<td>CK (U/liter)</td>
<td>72–404</td>
<td>355 ± 214</td>
<td>608 ± 666</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are the number, out of 3 horses, with results out of the normal range.
† Out of 31 analytes, those that were out of normal ranges for animals in these experimental conditions.
‡ Mean ± 2 SD, calculated from values for the control group (horses treated with ground alfalfa) and preexposure samples of the test groups.
§ Significantly different than control animals on that sample date (P < 0.05).
had low thrombocyte counts (80,000 and 5,000 platelets/µl) prior to euthanasia. Biochemically, the high-dose animals had several significant serum changes (Table 1). As individual animals became ill, mean serum SDH, bile acids, ALP, and bilirubin (total, direct, indirect) levels increased. Other changes indicative of decreased hepatic function included decreased GLU and BUN concentrations and increased BSP retention times, NH$_3$ concentrations, and coagulation times (Table 1). There were other sporadic biochemical changes, but values for these parameters were not significantly different ($P > 0.05$) from those of controls and low-dose animals on those sample dates (Table 1).

The low-dose animals had sporadic increases in bile acids, GGT, and SDH at times that corresponded to the biopsy findings of hepatocellular degeneration and necrosis (days 28-63). These alterations were less frequent later in the study (days 70-168). Although these increases were higher than those found in nontreated normal animals, none of the group means were statistically different from control means at any of the sample times.

At necropsy, 2 of the high-dose animals had cecal or ileal infarctions, and all 3 animals had extensive cecal and colonic mucosal edema (Fig. 3). All 3 high-dose horses had soft, red, wet livers with prominent lobular patterns. One animal had severe necrotizing dermatitis with extensive crust formation on the nose, face, and thorax where the hair was thin or white (Fig. 2). No significant gross lesions were noted in the low-dose or control animals.

Histologically, by day 28 the high-dose horses developed hepatic lesions typical of acute pyrrolizidine toxicosis. Changes included extensive hepatocellular necrosis with mild periportal fibrosis and biliary hyperplasia (Fig. 4). Some necrotic hepatocytes formed syncytia, but there was no megalocytosis. At 28 days, hepatic changes in the low-dose animals included mild, patchy hepatocellular necrosis, biliary hyperplasia, and focal neutrophilic periportal inflammation (Fig. 5). Between days 56 and 168, these lesions resolved, however, after week 30 all 3 low-dose animals had increased hepatocellular apoptosis, anisokaryosis, and megalocytosis (Fig. 6). No histologic or cytologic alterations were found in the control animals.

On day 14, the high-dose horses had sporadic increases in PCNA-positive hepatocytes, biliary epithelial cells, and inflammatory cells. These same animals had even more immunoreactive cells on day 28; however, mean differences were not statistically significant (Fig. 7). The low-dose horses also had increased numbers of PCNA-positive cells that were primarily biliary epithelium. The number of immunoreactive cells re-
Figure 4. Photomicrograph of the liver of a horse that was dosed with houndstongue containing 15 mg/kg total pyrrolizidine alkaloid for 14 days and was necropsied on day 28. Note the extensive hepatocellular necrosis (n), hepatocyte syncytia (s), and peribiliary (b) inflammation (*). HE, bar = 50 µm.

Figure 5. Photomicrograph of the liver of a horse that was dosed with houndstongue containing 5 mg/kg total pyrrolizidine alkaloid for 14 days. This biopsy was taken on day 28. Note the focal hepatocellular necrosis/apoptosis (n) and biliary epithelium and ovalocyte hyperplasia (b). HE, bar = 25 µm.
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Figure 6. Photomicrograph of the liver of a horse that was dosed with houndstongue containing 5 mg/kg total pyrrolizidine alkaloid for 14 days. This biopsy was taken on day 168. Note the megalocytosis (m) with less prominent peribiliary inflammation (b). HE, bar = 50 µm.

remained high and was significantly higher than that of control animals on days 42, 56, and 112 (Fig. 7).

There was no difference in hepatocyte nuclear areas between the high-dose animals and controls. Small numbers of the hepatocyte nuclei of the low-dose horses were very large (Fig. 6), and the mean nuclear areas were significantly different from those of controls on days 56, 70, and 84 (Fig. 8a). The increase in mean nuclear area was due to the presence of megalocytes (Fig. 8b), as the average group deviation also increased at those times.

Pyrroles were detected in hepatic tissue from all the high-dose horses by GC/MS and the spectrophotometric detection of the reaction with Ehrlich’s reagent (Fig. 9). A trace amount of pyrrole was sporadically detected in liver from 1 of the low-dose horses. Pyrroles were never detected in liver from control animals.

Discussion

There are several reports of houndstongue toxicosis in livestock (Table 2). One aged pony was dosed with 360 mg/kg (total PA) for 20 days (7,200 mg total dose) with no significant clinical or biochemical changes. In the present study, houndstongue was much more toxic. All 3 horses dosed with 15 mg/kg total PA for 14 days (total PA dose of 223 ± 5 mg/kg) developed severe toxicoses, with gross and microscopic lesions consistent with acute PA intoxication. This large disparity in reported toxicities may be due to the age of the animals. Young animals are much more susceptible to PA toxicity. The difference may also be due to the composition of the PA. The houndstongue fed to these

| Table 2. Relative toxicity of houndstongue (Cynoglossum officinale) and its alkaloids. |
|-----------------------------------------------|-----------------|-----------------|
| Agent                                        | Dose (mg/kg)    | Reference no.   |
| Houndstongue (total PA)                      |                 |                 |
| Gavaged daily to horse for 20 days           | 360 (nontoxic)  | 9               |
| Houndstongue (total PA)                      |                 |                 |
| Gavaged to calves once                        | 60              | 1               |
| Gavaged daily for 21 days                    | 15              | 1               |
| Heliosupine (pure)                           |                 |                 |
| Intraperitoneally in rats                     | 60              | 3               |
| Acetylheliosupine (pure)                     |                 |                 |
| Intraperitoneally in rats                     | 60              | 3               |
| Echinatine (pure)                            |                 |                 |
| Intraperitoneally in rats                     | 350             | 3               |
| 7-Angelylheliotridine (pure)                 |                 |                 |
| Intraperitoneally in rats                     | 60              | 3               |
| Houndstongue (total PA)                      |                 |                 |
| Gavaged to horses for 14 days                | <15             | current report  |
Figure 7. Graph of cell proliferation rate (determined by immunohistochemistry with antibodies to proliferating cell nuclear antigen) in horses treated with houndstongue or alfalfa containing 0, 5, and 15 mg/kg total pyrrolizidine alkaloid for 14 days.

Figure 8. Graph of nuclear morphometry (a) and average standard deviation (b) in horses treated with houndstongue or alfalfa containing 0, 5, and 15 mg/kg total pyrrolizidine alkaloid for 14 days.

horses was 0.62% N-oxide. N-oxides are often less toxic; however, in most animals, especially ruminants, when N-oxides are given by mouth they are presumably reduced by the intestinal microflora into the free base, resulting in toxicity similar to that of the free base.\textsuperscript{12,18}

The concentrations of the different houndstongue PAs (heliosupine, acetylheliosupine, echinatine, 7-angelylheliotridine) is extremely variable, which may result in varying toxicity.\textsuperscript{23} Houndstongue is likely to be harvested with forages when it is most toxic; young, actively growing plants often have higher PA concentrations than do senescent plants.\textsuperscript{20} It has also been shown that Echinatine is only about 15% as toxic as acetylheliosupine and 7-angelylheliotridine (Table 2). Because proportions of individual alkaloids can fluctuate with growth stage, environmental conditions, and plant populations, considerable variation in toxicity levels for the total PA may occur.\textsuperscript{23} Thus both plant and animal factors may account for the relatively low toxicity found previously.\textsuperscript{9}

Based on the findings of this study, any level of houndstongue contamination of feed should be considered potentially lethal for all livestock. For example, assuming a total PA concentration of about 0.8% when houndstongue is likely to be harvested with forages, a 500-kg horse would have to eat about 380 g of dried plant for 2 weeks to develop clinical disease. This would be about 1 plant per day, which is about 2% of a horse’s daily intake, an amount easily attainable in infested pastures or hay. Recent studies suggest houndstongue may be equally toxic for cattle.\textsuperscript{1}

Many clinical signs seen in the horses treated with 15 mg/kg PA such as lethargy, anorexia, icterus, and photosensitivity are typical of acute liver disease.\textsuperscript{8} The low-dose horses also had transient anorexia with sporadic increases in serum enzymes and bile acids. None of these changes are specific for PA toxicosis. In fact, similar changes are commonly seen in horses as a result
of various infectious or metabolic diseases or simple fasting or anorexia.\textsuperscript{4}

In this study, the evaluation of hepatic biopsies was the most sensitive indicator of PA toxicosis. In the low-dose horses, histologic lesions compatible with PA toxicosis were present throughout the study. These changes initially included mild periportal hepatocyte necrosis and biliary epithelial proliferation with minimal periportal fibrosis. As the disease progressed, these lesions became less prominent and extensive megalocytosis developed. These changes correlated well with increased hepatocyte area and proliferation of the biliary epithelium (Figs. 7, 8).

The gross lesions of edema and infarction of the cecum and colon in the high-dose horses were dramatic (Fig. 3). Abomasal edema has been reported in PA-intoxicated cattle, but such extensive visceral edema has not been previously described in PA-poisoned horses.\textsuperscript{7} Because similar infarctions are often associated with vascular diseases, including verminous arteritis, without thorough postmortem examination some PA intoxications could be misdiagnosed. The exact pathogenesis of PA-induced vascular lesions is not yet known. Decreased intravascular osmotic pressure, mucosal edema, vascular stasis, or focal intravascular coagulation may result in thrombosis and infarction. Such coagulopathies could result in consumption of clotting factors, increased clotting times, and decreased fibrinogen concentrations. All of these changes occurred in the high-dose animals. Because anticoagulant factors such as antithrombin III are synthesized in the liver, PA-induced liver disease could cause decreased hepatic synthesis of these proteins, resulting in reduced anticoagulant activity and a hypercoagulable state.\textsuperscript{5} These vascular lesions may also be the result of a direct toxic effect. Various PAs, including monocrotaline, can damage the renal and pulmonary vasculature.\textsuperscript{25} Additional work is needed to determine if the pathogenesis of these vascular lesions is similar.

The hepatic lesions seen in these horses were very similar to those reported in horses fed \textit{Senecio} or \textit{Crotalaria} spp.\textsuperscript{10,16} Other studies have not emphasized that in acute PA hepatic disease, such as occurred in the high-dose horses, the lesions are not pathognomonic. Classically, hepatic changes of necrosis, biliary hyperplasia, fibrosis, and megalocytosis are considered indicative of PA toxicosis.\textsuperscript{5} The high-dose horses in this study had extensive hepatocellular necrosis with collapse of hepatic cords and sparing of portal structures (Fig. 4). Fibrosis and biliary epithelial hyperplasia were minimal. There was little or no true megalocytosis. Similar hepatic necrosis is seen in other diseases, including serum hepatitis, bacterial hepatitis, and other toxic hepatopathies.\textsuperscript{5} In comparison, megalocytosis, which is considered a classical histologic indicator of PA poisoning, was found only in the low-dose horses that had survived months with the disease. In these animals, megalocytosis was the most prominent change 6 months after exposure (Fig. 6).

Tissue-bound pyrroles were detected and extracted from the livers of all 3 high-dose horses using both GC/MS and Ehrlich’s reagent. Detection of pyrroles was qualitative in this study, and serial extractions of liver from the same horse often yielded different amounts of pyrrole. This technique, especially when the pyrrolic ether was detected with GC/MS, was specific (there were no false positives); however, it lacked sensitivity. Pyrrole was rarely detected in livers of the low-dose horses. In these animals, the pyrrole was probably metabolized or cleared to levels that are undetectable with these techniques. Although others have developed comparable techniques to detect PA metabolites, none have documented the sensitivity or related the results to pyrrole clearance.\textsuperscript{11,14} Additional work must be done to improve the sensitivity of these techniques or to develop an immunologic quantitative assay. This type of assay probably will be most suc-
cessful because it could detect pyrroles without the necessity of extracting them from tissues.

These findings suggest that 1) isolation and identification of tissue-bound pyrroles can be used to demonstrate exposure to PA-containing plants in most cases of acute PA toxicosis (acute toxicosis may be the most difficult to differentiate from other causes of hepatic necrosis); 2) houndstongue is much more toxic to horses than previously reported; and 3) acute houndstongue toxicosis causes marked biochemical changes and histologic hepatic necrosis without megalocytosis. Chronic or low-dose intoxication causes minimal biochemical changes with dramatic hepatocyte megalocytosis.

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Sources and manufacturers

a. Stack S Hematology Analyzer, Coulter Corp., Miami, FL.

b. Monarch plus, Instrumentation Laboratories, Lexington, MA, and Indiana Veterinary Diagnostic Laboratories, Indianapolis, IN.

c. ACL 1000, Instrumentation Laboratories, Lexington, MA, and Indiana Veterinary Diagnostic Laboratories, Indianapolis, IN.

d. PC10, DAKO Corp., Carpinteria, CA.

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