Comparison of ELISA and HPLC for the Determination of Histamine in Cheese

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A competitive direct enzyme-linked immunosorbent assay (CD-ELISA) for histamine in cheese was compared with a reversed-phase liquid chromatography (RP-HPLC) method. Cheese was homogenized with phosphate-buffered saline (PBS), centrifuged, and filtered, and the supernatant was diluted with PBS for CD-ELISA. For RP-HPLC, biogenic amines (histamine, tyramine, putrescine, and cadaverine) were derivatized with 9-fluorenylmethylchloroformate, followed by reversed-phase chromatography and fluorescence detection. Detection limits and mean recoveries (10−1000 mg/kg) were 2 mg/kg and 93% for CD-ELISA and 1 mg/kg and 99% for RP-HPLC, respectively. Analysis of 50 commercial cheeses according to both methods showed good agreement for histamine (r = 0.979; concentration range = 2−1800 mg/kg). At a threshold level of 10 mg/kg, the ELISA gave no false-negative and three false-positive results. The results show that the ELISA is suitable for the determination of histamine in cheese.

Keywords: *Biogenic amines; histamine; cheese; enzyme immunoassay; liquid chromatography*

INTRODUCTION

Biogenic amines such as histamine, tyramine, and others can be formed in foods as a consequence of the metabolic process of microorganisms. Next to certain fish species (Scombridae), major sources of dietary biogenic amines are several types of long ripened cheeses (Stratton et al., 1991). Histamine at levels usually exceeding 1000 mg/kg has been implicated with certain food intoxications such as scombrotoxicosis (Taylor et al., 1984) or the "cheese syndrome" (Taylor et al., 1982). Reported adverse effects of dietary histamine or other biogenic amines include hypotension (histamine, putrescine, cadaverine), hypertension (tyramine), and headache, nausea, and emesis (Edwards and Sandine, 1981). The critical dose of oral histamine has been estimated to be in the range of 100−200 mg (Lüthy and Schlatter, 1983; Treptow and Askar, 1996). In rats, the no-observed adverse effect level for tyramine, putrescine, and cadaverine was reported to be at 180 mg/kg of body weight and day (Til et al., 1997). Although the importance of dietary histamine as a potential risk for human health is still not proven and has been controversial in the past (Lüthy and Schlatter, 1983; Morrow et al., 1991; Clifford et al., 1991; Silla-Santos, 1996; Melnik et al., 1997), regulatory levels in the range of 50−200 mg of histamine/kg of fish tissue have been set by the European Union, the U.S. FDA, and several other countries (European Union, 1991; Fletcher et al., 1998). Despite the fact that cheese may contain exceedingly high levels of histamine and other biogenic amines (>2000 mg/kg), tolerances have not been set so far.

Several physicochemical methods for histamine, in particular fluorometry and liquid chromatography (LC), have been described, and so far these are the most commonly used routine assays for biogenic amines in foods in general (Stratton et al., 1991). These methods are usually costly, require extensive sample cleanup, and have a low sample throughput. To enhance food control measurements, and possibly to help clarify the role of dietary histamine for human health, rapid and easy analytical methods for this compound would be beneficial.

Most immunochemical methods (enzyme immunoassays) for the detection of histamine in human serum, biological fluids, and foods are based on antibodies against *N*-amino derivatives of histamine synthesized by reaction with, for example, *p*-benzoquinone or propionic acid esters (Chevrier et al., 1986; Guesdon et al., 1986; Peyret et al., 1986; Morel and Delaage, 1988; Hammar et al., 1990; Rauch et al., 1992; Krüger et al., 1995; Serrar et al., 1995). However, because the antibodies used in these tests are not reactive with the parent compound but with a histamine adduct, chemical derivatization of histamine is necessary before analysis, which either is time-consuming (propionic acid esters) or requires toxic reagents (*p*-benzoquinone). Several commercial tests based on such antibodies are commercially available (IBL GmbH, Hamburg, Germany; Transia Diagnostics, Ober-Mörlen, Germany). Recently, the first polyclonal anti-histamine antibodies recognizing intact histamine were prepared (Schneider et al., 1996) and incorporated in a commercial ELISA test kit (R-Biopharm GmbH, Darmstadt, Germany). Here we report the use of these antibodies in a CD-ELISA for the detection of histamine in cheese. The CD-ELISA

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results were evaluated by comparison with a reversed-phase (RP) HPLC method.

EXPERIMENTAL PROCEDURES

Chemicals. Histamine dihydrochloride, tyramine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, 3,3′,5,5′-tetramethylbenzidine (TMB), casein (sodium salt), and polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany. CD-ELISA enzyme substrate/chromogen solution (Gallati and Pracht, 1985) was H_2O_2 (3 mM) and TMB″ (1 mM) in potassium citrate buffer (0.2 M; pH 3.9). Derivatization solution (9-fluorenylmethylchloroformiate (FMOC) in acetone, 3.87 mg/mL) for LC analyses was from Grom Analytik + HPLC GmbH, Herrenberg, Germany.

Immunochemicals. Rabbit antiserum against histamine, produced by immunization with a histamine−keyhole limpet hemocyanin conjugate (prepared by glutaraldehyde reaction), was used as described earlier (Schneider et al., 1996). The serum was precipitated against ammonium sulfate (Hebert et al., 1973) and dialyzed against phosphate-buffered saline (PBS; pH 7.2−7.3, 0.01 M phosphate buffer containing 0.1 M NaCl). Histamine−horseradish peroxidase conjugate for use as the labeled antigen in CD-ELISA was prepared by reductive alkylation using sodium periodate (Wilson and Nakane, 1974) under reaction conditions as described by Schneider et al. (1996).

Sample Materials. Cheese samples (n = 50) were purchased from food shops in southeastern Germany (Munich and Kulmbach areas). These samples included a large variety of types of hard cheeses (n = 31; e.g., Emmental, Cheddar; some of them made from raw milk), semihard cheeses (n = 14; e.g., Gouda, Edam), and soft cheeses (n = 5; e.g., Camembert, Romadur). A 100−200 g portion of each sample was cut up in a blender at high speed for ∼2 min and stored at −18 °C until analysis.

Histamine Analysis by CD-ELISA. A 2 g portion of cheese was homogenized (Stomacher) with 10 mL of PBS (pH 7.2) in a plastic bag for 5 min. The mixture was transferred to a test tube and centrifuged (1500*g*, 4 °C, 15 min). A portion of the supernatant (0.2 mL) was diluted with 0.6 mL of PBS, ready for CD-ELISA analysis. CD-ELISA was performed as described by Schneider et al. (1996). In brief, the wells of a microtiter plate were incubated with 100 μL/well anti-histamine antiserum (diluted 1:1000 with carbonate−bicarbonate buffer, pH 9.6) overnight at ambient temperature. Free protein binding sites were blocked with a solution of 2% (w/v) sodium caseinate in PBS (150 μL per well) for 30 min. The plate was washed (distilled water containing 0.85 g of NaCl and 0.25 mL of Tween 20 per liter) three times and drained. Histamine standard or sample extract solution (in PBS; 50 μL/well) was then added, followed by histamine−HRP solution [in 1% (w/v) sodium caseinate/PBS; 50 μL/well]. After incubation for 2 h at room temperature, the plate was washed again, and enzyme substrate/chromogen solution (100 μL per well) was added. The enzyme reaction was stopped after 15 min with 1 M H_2SO_4 (100 μL per well), and the absorbance measured at 450 nm. All standard and sample extract solutions were analyzed in quadruplicate. The histamine content was quantified as described earlier (Usleber et al., 1994) with a competitive ELISA software (Märtlbauer, 1993), which uses a cubic spline function for calculation of the standard curve. The program also calculates the detection limit (Student's t, n = 4; 95% confidence limit) and the 50% inhibition dose. The measuring range of the standard curve usually is from 20 to 80% relative binding ($B/B_0 \times 100$).

Analysis of Biogenic Amines by RP-HPLC. A PBS extract of 2 g of cheese was prepared the same way as for CD-ELISA. Precolumn derivatization and LC determination were performed as described earlier (Maier-Rosenkranz et al., 1994). In brief, a portion of the supernatant (0.2 mL) was diluted with 1.0 mL of internal standard solution (1,7-diaminoheptane in water; 13.02 mg/mL water) in a glass test tube. Acetone (1.2 mL) was added and mixed; after 10 min, the mixture was centrifuged again. Twenty microliters of the supernatant was then mixed with 60 μL of borate buffer (0.5 M, pH 9.0) and 80 μL of FMOC solution. After 45 s, stop solution (glycine in water; 3 mg/mL) was added, and after 45 s, the reaction mixture was diluted with sodium acetate buffer (540 μL). Ten microliters of this extract was injected into the HPLC system (Kontron Instruments, Munich, Germany). The HPLC system consisted of two model 422-Master pumps, model 465 autosampler, model SFM fluorescence detector, and 450-MT/EMS data system. Chromatographic separation and determination of histamine, tyramine, putrescine, and cadaverine were performed using a GROM-SIL Polyamin 2 analytical column, 250 × 4 mm i.d. (Grom, Herrenberg, Germany); the mobile phase consisted of a stepwise gradient of (A) acetonitrile/water/acetic acid (100:400:3) and (B) acetonitrile/water/acetic acid (475:25:3) at a flow rate of 1 mL/min and a temperature of 40 °C. Derivatized amines were detected using the fluorescence detector set at an excitation wavelength of 265 nm and an emission wavelength of 310 nm.

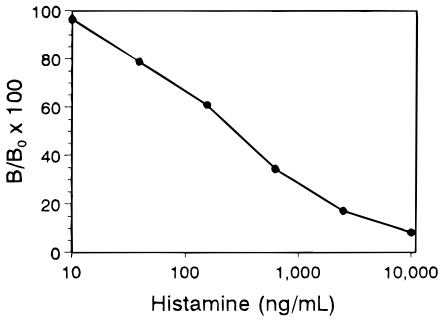

Figure 1. Typical standard curve of the CD-ELISA for histamine. Each point represents the mean of four determinations. B_0 was at 1.5 absorbance units. The intraassay and interassay RSD were usually 1.5−8 and 3−10%, respectively.

Table 1. Recovery of Histamine from Spiked Cheese (CD-ELISA and HPLC)

histamine added (mg/kg)	histamine found			
	mean (mg/kg)	RSD (%)	recovery (%)	n
	CD-ELISA			
10	10.5	22	105	4
20	18.3	30	91.5	3
100	85.9	18	85	5
1000	885	20	88	5
	HPLC			
10	9.8	13.2	98	4
100	97.9	5.4	97.9	4
1000	1003.2	2.2	100.3	4

RESULTS

After evaluation of 40 CD-ELISA standard curves performed over a period of 3 months, the mean 50% inhibition concentration and the mean standard curve detection limit were found to be at 210 ± 87 and 82 ± 49 ng/mL, respectively. A typical standard curve is shown in Figure 1. Considering a minimum sample extract dilution factor of 20 and an average recovery of 93% (Table 1), the detection limit for histamine in cheese was calculated to be at 2 mg/kg. The detection limit of the HPLC method (3× noise level) for histamine was at 1 mg/kg; recoveries are listed in Table 1.

Of 50 commercial cheese samples analyzed by HPLC, 30 contained histamine at levels ranging from 1.3 mg/kg to a maximum content of 1720 mg/kg in one cheese made from nonpasteurized milk; the mean of positives was 274 mg/kg. By CD-ELISA, 33 samples were positive in a range from 2.2 to 1840 mg/kg; the mean of positives was 255 mg/kg. For quantitative analysis of histamine, results obtained by using both methods (Figure 2) showed good agreement (r = 0.979). Table 2 shows a summarized comparison of the results obtained by CD-

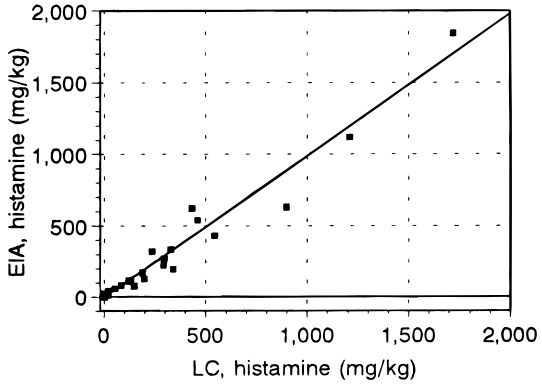

Figure 2. Comparison of LC and CD-ELISA results for histamine in cheese samples ($n = 50$; $r = 0.979$). The linear regression line is given by $y = 0.99x - 0.012$.

Table 2. Comparison of CD-ELISA and HPLC Results for Histamine in Commercial Cheese Samples ($n = 50$), Grouped into Classes According to the Respective HPLC Result

HPLC result for histamine (mg/kg)	no. of samples	CD-ELISA result, no. of samples with histamine levels (mg/kg) of			
		<10	10−<100	100−<1000	>1000
<10	26	23	3		
10−<100	7		7		
100−<1000	15		1	14	
>1000	2				2

Table 3. Results for Biogenic Amines (Milligrams per Kilogram) in Hard Cheese, Semihard Cheese, and Soft Cheese

	cheese type		
	hard cheese ($n = 31$)	semihard cheese ($n = 14$)	soft cheese ($n = 5$)
	Histamine (HPLC)		
n positive	22	4	4
mean	352	34	78
median	216	6	8
max	1720	122	296
	Histamine (CD-ELISA)		
n positive	24	4	4
mean	322	33	73
median	148	8	8
max	1840	113	270
	Tyramine (HPLC)		
n positive	23	7	4
mean	173	78	164
median	109	55	147
max	520	220	324
	Putrescine (HPLC)		
n positive	18	7	5
mean	74	73	179
median	46	32	140
max	254	282	441
	Cadaverine (HPLC)		
n positive	21	7	5
mean	123	15	234
median	61	3	62
max	254	80	635
	Total Biogenic Amines (HPLC)		
n positive	29	9	4
mean	522	93	506
median	358	5	215
max	2250	705	1700

ELISA and HPLC in commercial cheese samples, grouped according to the HPLC result. The mean levels of histamine, tyramine, cadaverine, and putrescine in cheese samples are listed in Table 3. The relationship between histamine levels and the total concentration of biogenic amines is shown in Figure 3.

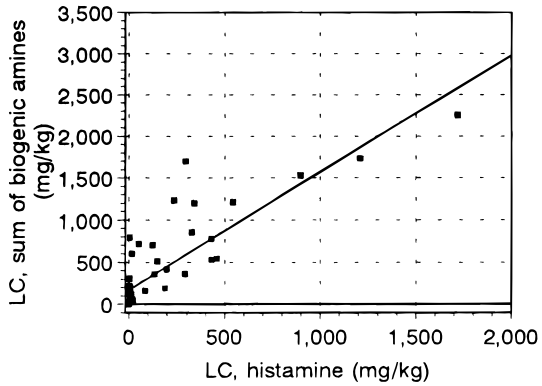

Figure 3. Comparison of LC results for histamine and the sum of biogenic amines (histamine, tyramine, putrescine, cadaverine) in cheese samples ($n = 50$; $r = 0.848$). The linear regression line is given by $y = 1.40x + 169$.

DISCUSSION

With test sensitivities in the lower milligrams per kilogram range, both CD-ELISA and HPLC enabled detection of histamine in cheese at levels well below those that have to be considered to be relevant for human health (Treptow and Askar, 1996). Because nonpurified aqueous extracts could be analyzed, the maximum daily throughput of the CD-ELISA method is >30 cheese samples. The limiting factor of this study was the LC method, which requires laborious sample extract preparation and chromatographic run times of 50 min, limiting the daily sample throughput to <10 samples.

Differences between LC and CD-ELISA determinations of histamine were found almost exclusively for the low concentration range from 2 to 10 mg/kg. Assuming a maximum daily consumption of 100−200 g of cheese, histamine levels <10 mg/kg can be considered as negligible. At concentrations >10 mg/kg, agreement between both methods was excellent (Figure 2).

Because histamine levels of ∼100 mg/kg have to be considered as critical and levels >1000 mg/kg are potentially toxic, respectively (Treptow and Askar, 1996), the cheese samples were grouped according to their histamine concentration (as determined by HPLC), using ranges of <10, 10−100, 100−1000, and >1000 mg/kg, respectively (Table 2). Within this classification, no false-negative results were obtained by CD-ELISA; 46 of 50 samples were scored correctly by CD-ELISA, and 3 samples were slighly overestimated. One sample of hard cheese (150 mg/kg by HPLC) was underestimated by CD-ELISA (76 mg/kg). Therefore, the CD-ELISA method can be considered as a reliable method for the quantitative determination of histamine in cheese at levels >10 mg/kg.

In addition to histamine, the levels of three other biogenic amines (tyramine, putrescine, and cadaverine) commonly found in cheese (Kielwein, 1996) were determined by HPLC. The concentrations found for these amines (Table 3; Figure 3) show that their sum frequently exceeds a level of 1000 mg/kg. As reported by many authors before [for reviews see, e.g., Stratton et al. (1991) and Kielwein (1996)], hard cheeses had the highest overall content of histamine, tyramine, putrescine, and cadaverine, although some semihard cheeses and soft cheeses also contained considerable concentrations of these compounds. Most positive samples contained all four amines, which is reflected by the fact that only eight samples contained none of these sub-

stances. Therefore, an interesting question was whether the histamine concentrations in cheese could be used as an indicator for elevated levels of total biogenic amines.

Although there was no quantitative correlation between histamine and any of the other compounds alone ($r < 0.2$), the sum all four biogenic amines was strongly related ($r = 0.848$) to the histamine content (Figure 3). All samples that contained >1000 mg/kg of total biogenic amines had histamine levels of at least 100 mg/kg. Additionally, the highest tyramine levels (>200 mg/kg) were found only in samples that exceeded histamine concentrations of 100 mg/kg. This could be important because tyramine has been implicated in adverse reactions involving headache and hypertensive crisis in patients taking monoamine oxidase inhibitors (Stratton et al., 1991). Although the number of samples under study was too small to derive any definite conclusion from these findings, the data still indicate that the histamine level as obtained by CD-ELISA could be used as an indicator for the cumulative contamination level with biogenic amines. Further analyses of a larger number of cheese samples are required to verify these results.

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