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High-performance liquid chromatographic determination of biogenic amines in fish implicated in food poisoning

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Abstract

A rapid, sensitive and reproducible high-performance liquid chromatographic procedure for the determination of nine biogenic amines in fish by improved benzoylation with benzoyl chloride was developed. The benzoylation of amines with benzoyl chloride at 30°C for 40 min was the optimal condition to eliminate the influence of interfering peaks during analysis. The calibration curve for each amine was linear within the range of 0.02–4 μg. The amine recovery from fish meat was better by extraction with 6% trichloroacetic acid than with 1 *M* $HClO_4$. The application of this method to detect amines in a fried marlin fillet implicated in a food poisoning incident indicated that a high level (84.1 mg/100 g) of histamine was present in the sample.

Keywords: Amines; Histamine

1. Introduction

Biogenic amines are widely present in all living cells and a variety of foodstuffs [1–3]. Some amines, such as putrescine, cadaverine, spermidine and spermine, have been implicated in growth processes, particularly in the proliferation of eukaryotic cells [4,5]. Among all of the biogenic amines that occur in foods, histamine is potentially hazardous and is a causative agent in scombroid fish poisoning [6]. Furthermore, biogenic amines have been found to be useful as quality indices for the decomposition of fish [7,8]. Hence, research on the simultaneous analysis of various biogenic amines in living cells and foods is of interest and importance.

Many chromatographic methods, especially high-performance liquid chromatography (HPLC), have been developed and widely used in the determination of the concentration of biogenic amines [8–12]. Among them, amine derivatization with fluorogenic agents can give specific and sensitive determination of amines. However, the reaction products have a short life and the method requires the preseparation of amines before derivatization [10]. The use of tosyl, dansyl or benzoyl chloride is preferred as they can derivatize most of the naturally occurring amines and form more stable reaction products. Due to the lengthy derivatization procedure with tosyl chloride, and the extremely long elution time required with dansyl derivatives, the use of benzoyl derivatives is advantageous [12].

A study [13] on the formation of biogenic amines in saifish, *Istiophorus platypterus*, responsible for scombroid poisoning has been conducted in our

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laboratory. The saifish samples were analyzed for amines using a gradient elution system of HPLC according to the method of Yen and Hsieh [14]. Although ten amines could be well separated by this method, interfering peaks were found occasionally with HPLC. Therefore, the present work is conducted to eliminate the influence of interfering peaks in HPLC analysis for amines in fish. The optimal conditions for derivatization of amines with benzoyl chloride and their simultaneous determination by reversed-phase HPLC are also studied. In addition, the technique was used to detect biogenic amines in a fried fillet of marlin (*Makaira mazara*) that was implicated in a food poisoning outbreak that occurred in Taipei City in April 1996.

2. Experimental

2.1. Reagents

Standard amines including tryptamine hydrochloride, 2-phenylethylamine hydrochloride, putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, histamine dihydrochloride, tyramine hydrochloride and agmatine sulfate, were obtained from Sigma (St. Louis, MO, USA). Methanol (LC grade) and other reagents (GR grade) were from Merck (Darmstadt, Germany).

2.2. Samples

The fresh frozen fillet of marlin (Makaira mazara, 336 g) was collected from a supermarket and the fried marlin fillet, implicated in a scombroid poisoning outbreak that caused illness in three young nurses, was obtained from an eating house. Both samples were wrapped and kept at −20°C until analysis.

2.3. Preparation of standard amine solution

Tryptamine hydrochloride (61.4 mg), 2-phenylethylamine hydrochloride (65.1 mg), putrescine dihydrochloride (91.5 mg), cadaverine dihydrochloride (85.7 mg), spermidine trihydrochloride (87.7 mg), spermine tetrahydrochloride (86.0 mg), histamine dihydrochloride (82.8 mg), tyramine hydrochloride (63.4 mg) and agmatine sulfate (87.7 mg) were dissolved in 50 ml of a 0.1 *M* HCl solution and used as the working solution. The final concentration of each amine (free base) was 1.0 mg/ml.

2.4. Determination of optimal reaction temperature and time for amine benzoylation

To 2 ml of mixed amine solution containing 0.1 mg of each amine, 1 ml of 2 *M* sodium hydroxide was added, followed by 10 μl of benzoyl chloride. The solution was mixed by using a vortex mixer and was allowed to stand at 20, 30, 40 and 60°C for 20, 40 and 60 min. The benzoylation was stopped by adding 2 ml of saturated NaCl solution, and the solution was extracted with 3 ml of diethyl ether. After centrifugation, the upper organic layer was transferred into a tube and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 1 ml of methanol and 20-μl aliquots were injected for HPLC analysis. The interfering peaks of remaining benzoyl chloride (amine-free) were also determined by using the same HPLC analysis procedure. The optimal reaction temperature and the time for amine benzoylation were obtained by the responding peak heights of amine and remaining benzoyl chloride in HPLC analysis.

2.5. Determination of the calibration curve of standard amine

To 2 ml of mixed standard amine solutions containing 0–0.5 mg of each amine, 1 ml of 2 *M* sodium hydroxide was added, followed by 10 μl of benzoyl chloride. The reaction temperature and the time were set at 30°C for 40 min, respectively. After benzoylation, the standard sample was extracted and determined by HPLC analysis as described in Section 2.4.

2.6. Separation of amines by HPLC

Amines were determined by using an Hitachi liquid chromatograph (Hitachi, Tokyo, Japan) consisting of a Model L-6200 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV–Vis detector, set at 254 nm, and a Model

D-2500 Chromato-integrator. A Lichrospher 100 RP-18 reversed-phase column (5 μm, 125×2.5 mm I.D., Merck) was used for the separation. The gradient elution program was set at 0.8 ml/min, starting with a methanol–water mixture (50:50, v/v) for 0.5 min. The program proceeded linearly to methanol–water (85:15, v/v), with a flow-rate of 0.8 ml/min over 6.5 min. This was followed by the same composition and flow-rate for 5 min, then a decrease over 2 min to methanol–water (50:50, v/v) at 0.8 ml/min.

2.7. Recovery of amines from a fish sample

After partial thawing, the fresh and fried marlin fillets were separately ground in a Waring blendor for 3 min. Each ground sample (5 g) was transferred to a 50-ml centrifuge tube, mixed with 1 mg of each standard amine and separately homogenized with 20 ml of 6% trichloroacetic acid (TCA) and 1 *M* $HClO_4$ for 3 min. The homogenate was centrifuged (8000 *g*, 10 min, 4°C) and filtered through Whatman No. 2 filter paper. The filtrate was placed in a volumetric flask and made up to 50 ml. Each extract (2 ml) was derivatized with benzoyl chloride using the same procedure as for the benzoylation of standard amine solutions. The recovery of amine in marlin meat was thus determined.

3. Results

The chromatographic profile of nine authentic biogenic amines by the gradient elution system was developed (Fig. 1). All nine amines were well separated in a total duration of 15 min, with good peak resolution, sharpness and symmetry. The interfering peaks of remaining benzoyl chloride in the HPLC profile are shown in Fig. 2. One interfering peak exhibited the same retention time (t_R=7.9 min) of tryptamine and the other one showed a retention time (t_R=11.3 min) that was between those of histamine and tyramine.

The effects of the temperature and duration of the

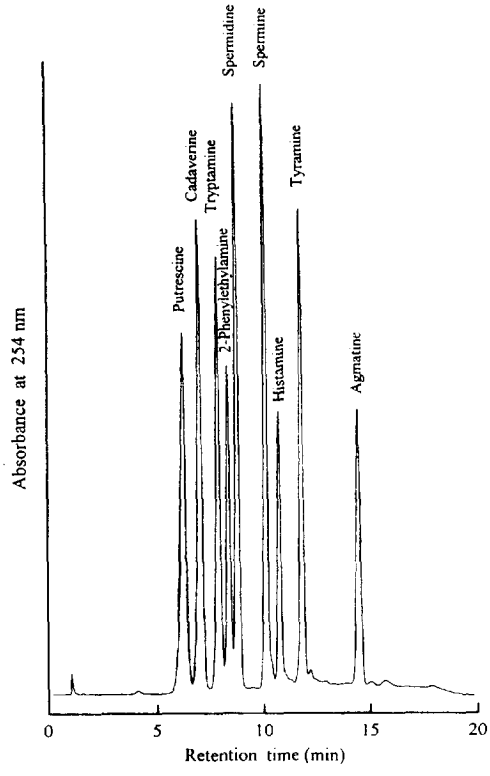

Fig. 1. HPLC profile of authentic amines.

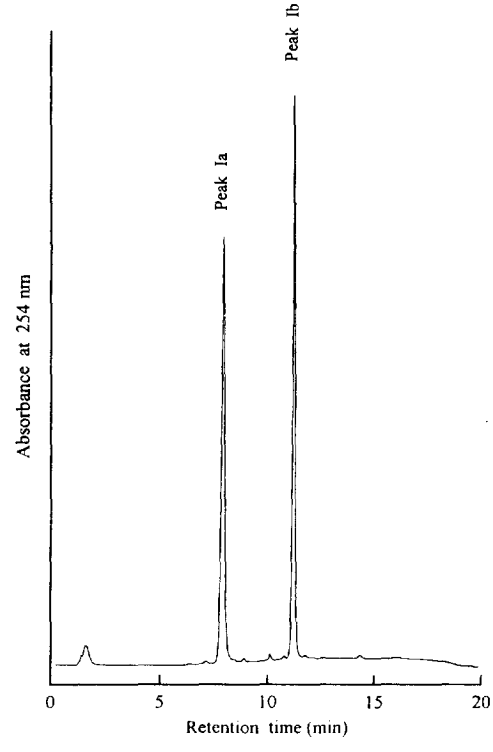

Fig. 2. HPLC profile of interfering peaks from remaining benzoyl chloride.

Table 1
Effects of temperature and time of benzoylation on the sensitivity of HPLC analysis for biogenic amines

Time of benzoylation (min)	Peak height of amines (absorbance at 254 nm)																	
	Put[a]		Cad		Tpm		Phe		Spd		Spm		Hm		Tym		Agm	
	Mean[b]	C.V.[c]	Mean	C.V.	Mean	C.V.	Mean	C.V.	Mean	C.V.	Mean	C.V.	Mean	C.V.	Mean	C.V.	Mean	C.V.
20°C																		
20	69 342	10.43	93 108	10.19	201 436	11.62	75 133	2.82	119 483	12.35	131 412	14.03	61 242	24.58	85 555	6.47	45 330	18.76
40	62 526	9.44	85 391	8.74	155 194	8.91	68 692	6.55	124 577	9.58	126 412	12.55	51 538	20.69	76 567	10.23	41 358	16.98
60	68 032	7.24	88 906	7.56	129 113	6.99	68 545	3.15	120 699	11.48	125 787	7.16	47 693	14.31	81 047	5.51	37 582	10.81
30°C																		
20	63 630	12.52	85 278	13.64	163 925	13.41	65 920	6.93	87 893	21.69	100 848	18.65	65 207	25.54	97 341	11.52	47 747	17.89
40	66 681	5.35	90 839	3.28	90 124	4.62	65 180	3.86	101 133	9.52	109 573	6.31	67 272	7.41	79 054	8.25	54 107	8.47
60	51 786	10.59	69 994	11.65	79 643	11.96	58 489	5.47	74 623	20.20	89 168	15.55	58 703	17.22	65 054	14.85	48 216	14.89
40°C																		
20	52 940	10.82	72 618	11.84	101 467	9.37	71 750	6.64	78 307	13.08	65 120	15.64	59 476	19.92	71 046	14.45	54 696	16.94
40	49 278	11.47	69 264	14.36	76 492	12.74	62 799	7.56	84 110	16.19	74 888	21.59	50 814	20.46	64 660	13.75	38 110	16.48
60	46 764	7.85	66 794	10.97	60 811	8.58	55 559	10.04	91 777	10.46	88 163	13.12	48 322	19.35	58 267	11.85	26 292	33.53
60°C																		
20	52 611	17.16	75 381	14.83	81 463	11.35	59 000	10.03	110 163	12.39	111 208	13.56	53 161	19.69	59 246	25.11	33 961	28.33
40	49 425	9.06	70 456	9.68	69 538	7.21	57 709	5.52	95 232	10.54	89 972	11.81	51 584	10.22	61 269	7.02	28 100	12.85
60	41 799	14.70	61 910	4.04	43 678	9.87	52 216	1.73	79 608	7.92	71 698	11.79	44 176	22.32	47 518	14.00	26 407	36.91

[a] Put=putrescine; Cad=cadaverine; Tpm=tryptamine; Phe=2-phenylethylamine; Spd=spermidine; Spm=spermine; Hm=histamine; Tym=tyramine and Agm=agmatine.

[b] $n=6$.

[c] C.V. means coefficient of variation and C.V.=(S.D./mean)×100%.

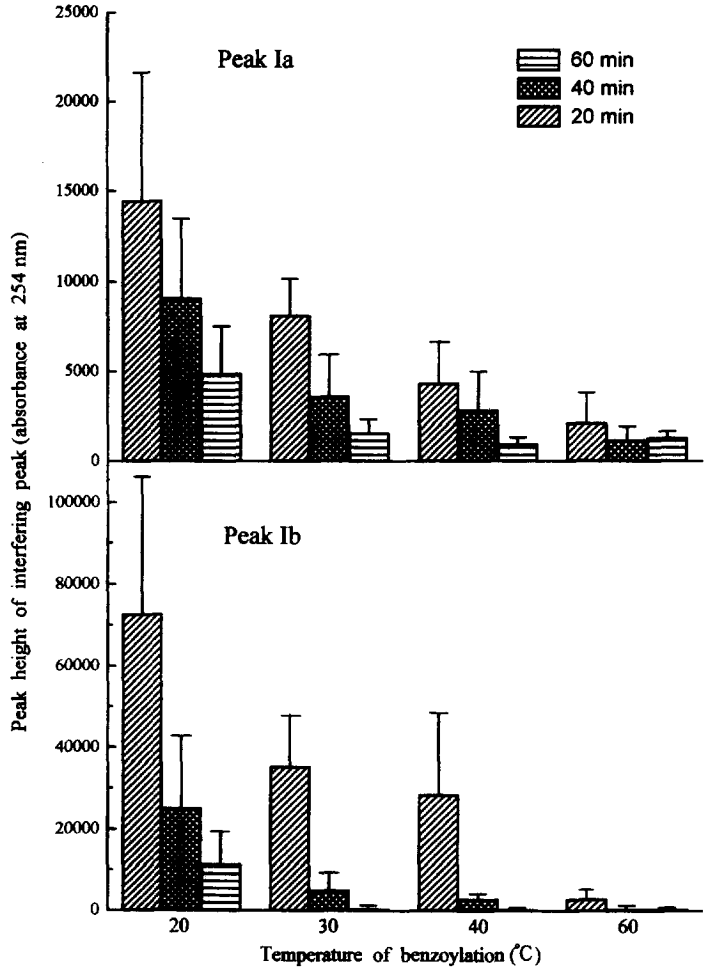

Fig. 3. Effects of benzoylating temperature and time on the interfering peak height ($n=6$).

benzoylation procedure on the sensitivity of HPLC analysis for biogenic amines are shown in Table 1. To gain a good responding peak height and coefficient of variation (C.V.) for each amine in the HPLC analysis, the optimal benzoylation conditions were found to be 30°C for 40 min. Amines benzoylated at 20°C for 20 min had a better peak height, but the C.V. was too large, indicating poor reproducibility. The effects of the temperature and duration of the benzoylation reaction on the interfering peak height of remaining benzoyl chloride in HPLC analysis are shown in Fig. 3. It was found that the interfering peak height was decreased with increasing benzoylation temperature and time. Judging from data in Table 1 and Fig. 3, the benzoylation of amines with benzoyl chloride at 30°C for 40 min is optimal for eliminating the influence of interfering peaks during HPLC analysis.

The detectable level of biogenic amines after benzoylation using the HPLC analysis system is shown in Fig. 4. The maximal detectable level was over 10 μg (0.25 mg/ml) for tryptamine and 2-phenylethylamine; 8 μg for histamine, tyramine and agmatine; 6 μg for putrescine and cadaverine; and 4 μg for spermidine and spermine. The calibration curve for each amine was linear within the range of 0.02–4 μg. Therefore, the standard curves of nine amines were separately prepared in the range 0–0.05 mg/ml, and peak height vs. amount of amine was plotted. Data for standard curves were subjected to

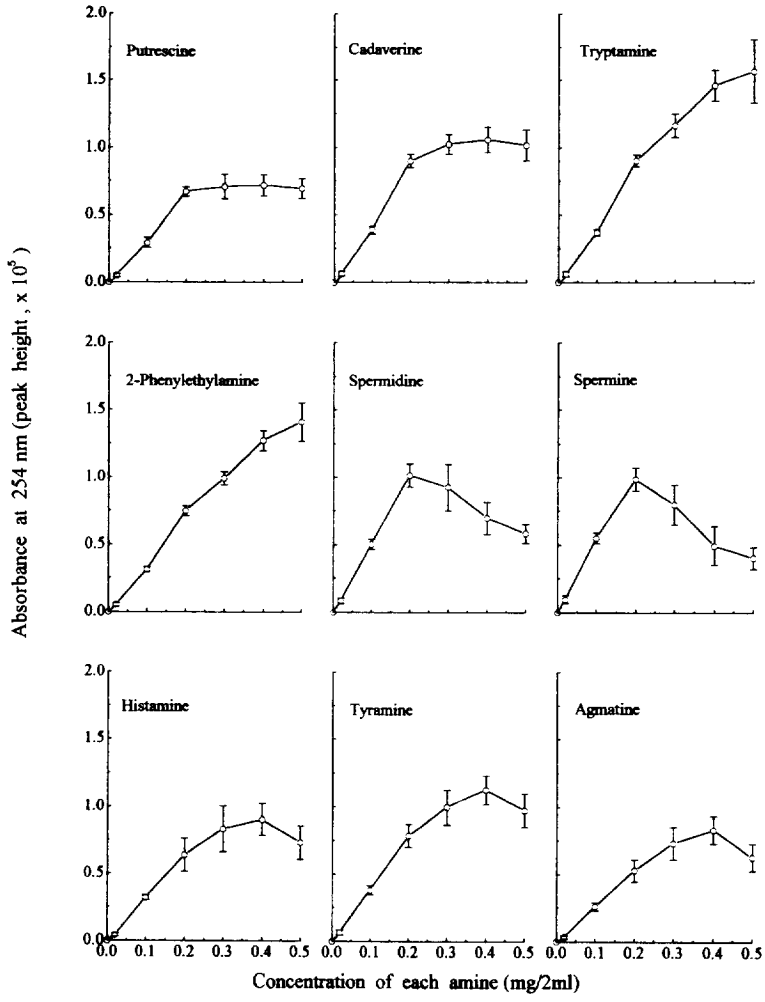

Fig. 4. Absorbance of different concentrations of amines derivatized with benzoylchloride at 30°C for 40 min and analyzed by HPLC.

linear regression analysis. The correlation coefficients and the linear regression coefficients for each amine were compared (Table 2). The correlation coefficient in every curve was 0.99. This indicated a definite linear relationship between amine concentration and detector response. Thus, the gradient elution program used in this study was satisfactory.

The recoveries of biogenic amines from fish meat were 101.2 and 99% for phenylethylamine, 100.5 and 100.2% for histamine, 98.2 and 98.0% for tryptamine, 76.5 and 62.4% for putrescine, 70.0 and 55.8% for cadaverine, 63.6 and 50.9% for spermidine, 63.0 and 33.0% for spermine, 35.0 and 101.2% for tyramine, and 4.5 and 3.0% for agmatine, by extracting with a 6% TCA solution and 1 *M* $HClO_4$, respectively ($n=6$). It was found that, with the exception of tyramine and agmatine, the other seven amines exhibited better recovery by extraction with 6% TCA solution rather than with 1 *M* $HClO_4$.

A typical HPLC profile of amines from a fried marlin fillet that was implicated in a food poisoning incident is shown in Fig. 5. The major amine levels in this sample were as follows: histamine, 84.1±2.6 mg/100 g; cadaverine, 8.5±0.4 mg/100 g and tryptamine 1.6±0.2 mg/100 g ($n=3$). The levels of other amines was less than 1 mg/100 g.

Table 2
Linear regression equation[a] correlation coefficient between amine concentration and absorbance in HPLC

Amine	Linear equation		Correlation coefficient
	a	*b*	
Putrescine (Put)	−23	35 635	0.99830
Cadaverine (Cad)	101	46 097	0.99877
Tryptamine (Tpm)	515	42 378	0.99914
2-Phenylethylamine (Phe)	−111	31 012	0.99964
Spermidine (Spd)	1157	52 196	0.99886
Spermine (Spm)	1466	53 755	0.99905
Histamine (Hm)	−1288	30 864	0.99827
Tyramine (Tym)	937	40 074	0.99877
Agmatine (Agm)	190	20 221	0.99855

[a] $y=a+bx$, where y=relative peak height and x=amount of amine injected into the HPLC system (0–2 μg).

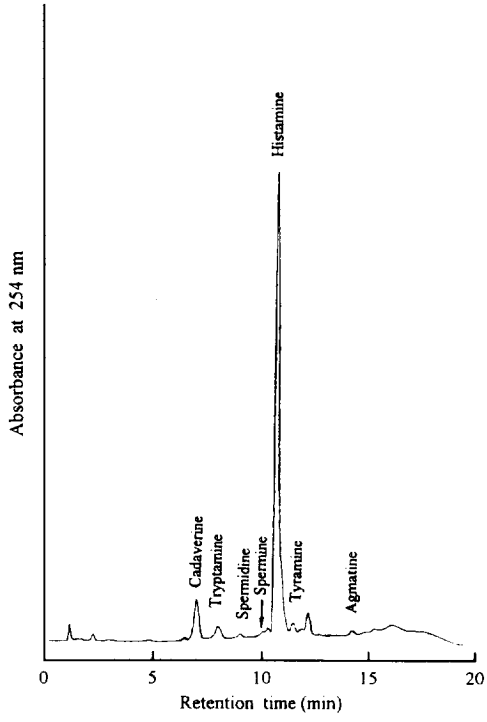

Fig. 5. HPLC profile of the amines present in a fried marlin fillet that implicated in a food-poisoning incident.

4. Discussion

It is well known that the concentration of histamine is considered to be a good indicator of fish deterioration and scombroid poisoning [15,16]. The U.S. Food and Drug Administration (FDA) has established a hazard action level of 50 mg of histamine/100 g of fish, based on data collected from numerous outbreaks [17]. Thus, the allergy-like symptoms of the victims, along with the high concentration of histamine in the fried marlin fillet, indicated that the food poisoning incident that occurred in Taipei may have been caused by histamine.

The production of histamine in fish is usually associated with spoilage. In fish, several histamine-producing bacteria, such as *Morganella morganii*, *M. vulfaris* and *Klebsiella pneumoniae*, have been implicated as primary contributors in histamine formation [18–20]. Due to the marlin fillet being fried, the causative bacteria in this scombroid poisoning incident might have been killed and was not isolated. About 90% of the biogenic amines were recovered from tuna and mackerel after sterilization [21]. The high level of histamine present in the fried marlin fillet seems to have originated from the poor quality of the raw fish before heating.

In this study, a rapid, sensitive and reproducible HPLC procedure was developed for the determination of nine biogenic amines in fish using benzoylation with benzoyl chloride. The optimal benzoylation conditions for biogenic amines was incubation at 30°C for 40 min, and the detectable level for the linear calibration curve is 0.02–4 μg for each amine. The recovery of biogenic amines from fish meat was better by extraction with 6% TCA than with 1 *M* $HClO_4$. Of the nine biogenic amines in fish, tyramine and agmatine showed the lowest recovery on extraction with 6% TCA. An efficient method for the extraction of these two amines from fish samples should be investigated further.

Acknowledgments

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