Development of High-Performance Liquid Chromatographic Method for Analysis of Histamine Released from Mast Cells

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ชื่อวิทยานิพนธ์	การพัฒนาวิธีวิเคราะห์สารฮีสตามีนที่หลั่งมาจากมาสท์เซลล์โดยใช้วิธีโครมาโตกราฟีของเหลวสมรรถนะสูง
ผู้เขียน	นางสาวมนชนา จุลลางกูร
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ปีการศึกษา	2551

บทคัดย่อ

การวิจัยนี้เป็นการพัฒนาวิธีวิเคราะห์เพื่อหาปริมาณสารฮีสตามีนโดยเทคนิคโครมาโตกราฟีของเหลวสมรรถนะสูง (HPLC) โดยใช้ตัวตรวจวัดชนิด ฟลูออเรสเซนต์ ขั้นตอนการวิเคราะห์ประกอบด้วย การ pre-column derivatization โดยใช้ *o*-phthalaldehyde (OPA) เป็น derivatizing agent แยกสารโดย ODS-C18 column และ mobile phase ที่เป็นสารผสมของ phosphate buffer, pH 6.4 (ที่มี 0.1%v/v TEA ผสมอยู่) : acetonitrile : methanol ในสัดส่วน 60 : 25 : 15 %v/v/v และ isocratic ที่มีอัตราการไหล 1 มิลลิลิตรต่อนาที ตรวจวัดโดยตัวตรวจวัดชนิดฟลูออเรสเซนต์ที่ความยาวคลื่น 348 และ 444 นาโนเมตร สำหรับ excitation และ emission wavelength ตามลำดับ วิธีวิเคราะห์ที่ได้พัฒนานี้ใช้เวลาในการวิเคราะห์ 18 นาทีต่อ 1 ตัวอย่าง, มีความจำเพาะเจาะจงโดยแสดงด้วยค่า %recovery 97.3 - 99.1%, ความแม่นยำที่แสดงโดย withih-run ที่มีค่า 25.05±0.28, 95.02±8.69 และ 201.08±1.42 และ between-run analysis ที่มีค่า 24.47±0.62, 99.76±7.49 และ 197.08±3.76 และมีค่า LOD, LOQ เท่ากับ 5 และ 10 นาโนกรัมต่อมิลลิลิตร ตามลำดับ

วิธีวิเคราะห์ที่ได้พัฒนาขึ้นนี้ได้ถูกนำมาวิเคราะห์ฮีสตามีนที่หลั่งจากมาสท์เซลล์ที่ถูกกระตุ้นด้วย compound 48/80 เพื่อศึกษาฤทธิ์ของสารสกัดจากเปลือกผลทับทิมในการยับยั้งการหลั่งฮีสตามีน จากการศึกษาพบว่า ค่า IC_{50} ของสารสกัด ellagic acid จากเปลือกผลทับทิมมีค่า 87.09 ไมโครกรัมต่อมิลลิลิตร

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ABSTRACT

An analytical method for quantitative determination of histamine was developed and validated. Following pre-column derivatization with *o*-phthalaldehyde (OPA), the obtained derivative was analyzed using an ODS-C18 column, and mobile phase system consists of phosphate buffer pH 6.4 (0.1%v/v TEA) : acetonitrile : methanol 60 : 25 : 15 %v/v/v. Fluorescence detection was employed at excitation wavelength 348 nm and emission wavelength 444 nm with isocratic elution at flow rate 1 ml/min, the total analysis time is 18 min. Specificity and accuracy was demonstrated with %recovery was in the range of 97.28 and 99.15%. Within-run and between-run analysis 24.47±0.62, 99.76±7.49, 197.08±3.76 and 24.47±0.62, 99.76±7.49, 197.08±3.76, respectively. LOD and LOQ was 5 and 10 ng/ml, respectively.

The method was used for determination of histamine released from rat mast cells activated by compound 48/80. Histamine release was found to be inhibited by the extract from *Punica granatum* peels (containing ellagic acid) in concentration dependent manner with an IC_{50} 87.09 µg/ml.

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Monchana Jullangkoon

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LIST OF ABBREVIATIONS AND SYMBOLS

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ECL	=	enterochromaffin-like	1
RIA	=	radio enzymatic assay	2
GC	=	gas chromatography	2
LC/MS	=	liquid chromatography tandom mass spectroscopy	2
HPLC	=	high-performance liquid chromatography	2
AQC	=	6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate	2
µg/ml	=	microgram/mililitre	2
OPA	=	*o*-phthalaldehyde	2
ng/ml	=	nanogram/mililitre	2
MW	=	molecular weight	7
HDC	=	histidine decarboxylase	7
GI	=	gastrointestinal	7
NaF	=	sodium fluoride	10
SAMe	=	*S*-[^{3}H] adenosylmethionine	11
DnsCl	=	dansyl chloride	11
UV	=	ultraviolet	12
ml/min	=	mililitre/minute	12
ref.	=	reference	12
λ_{ex}	=	excitation wavelength	13
λ_{em}	=	emission wavelength	13
THF	=	trihydrofuran	13
ACN	=	acetonitrile	13
ODS	=	octadecylsilane	13
KH_2PO_4	=	potassium dihydrogen phosphate	13
H_2NaPO_4	=	sodium dihydrogen phosphate	14
TEA	=	triethylamine	17
PSE	=	4-(1-pyrene)butyric acid *N*-hydroxysuccinimide ester	18
DNBZ-Cl	=	3,5-dinitrobenzoyl chloride	18

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

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SHS	=	sodium heptanesulfonate	21
PCA	=	passive cutaneous anaphylaxis	22
GSS	=	Gahmi-Shini-San	22
GRJ	=	the gall of *Rhus javanica*	22
CIAE	=	*Cichorium intybus*	22
mg/kg	=	milligram/kilogram	22
WEPL	=	*Phellinus linteus*	23
DNP	=	anti-dinitrophenyl	23
ABWE	=	*Agaricus blazei* water extract	23
VRFE	=	the fruits of *V. rotundifolia* (L.) (Verbenaceae)	23
BGE	=	Buckwheat grain extract	23
RPMC	=	rat peritoneal mast cells	24
TKT	=	Tongkytang	24
GBIT	=	Gamibojungikgitang	24
XF	=	*Xanthii fructus*	24
BSA	=	albumin from bovine serum, lyophilized	26
$CaCl_2 \cdot 2H_2O$	=	calcium chloride dehydrate	26
$(CH_3)_2SO$	=	dimethylsulphoxide	26
$Na_2HPO_4 \cdot 2H_2O$	=	di-sodium hydrogen phosphate dehydrate	26
Na_2EDTA	=	ethylenediaminetetraacetic acid	26
$C_8H_{18}N_2O_4S$	=	HEPES [4-(2-hydroxyethyl)piperazine-1-thanesulonic acid]	26
$C_{19}H_{19}NOS \cdot C_4H_4O_4$	=	ketotifen fumarate salt	26
$C_6H_9N_3O_2$	=	L-histidine	27
$MgCl_2 \cdot 6H_2O$	=	magnesium chloride hexahydrate	27
2-ME (C_2H_6OS)	=	2-mercaptoethanol	27
CH_3OH	=	methanol	27
KCl	=	potassium chloride	27
NaCl	=	sodium chloride	27

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

			Page
$NaH_2PO_4 \cdot 2H_2O$	=	sodium dihydrogen phosphate dehydrate	27
DI water	=	de-ionization water	28
MT-HEPES-BSA	=	Modified Tyrode-HEPES-BSA buffer	28
mmol/l	=	millimole/litre	29
KRP	=	Krebs Ringer Phosphate	30
LOD	=	limit of detection	36
LOQ	=	limit of quantitation	36
R^2	=	coefficient of correlation	36
QC	=	quantitative concentration	36
RSD	=	relative standard deviation	37
SD	=	standard deviation	37
EA	=	ellagic acid rich extracts	38
IC_{50}	=	inhibition concentration at 50%	60

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Histamine is a biogenic amine derived from enzymatic decarboxylation of a natural amino acid, histidine (Douabale *et al*., 2003, Previati *et al., 2002*). It was synthesized and stored in mast cells. Mast cells are constituents of virtually all organs and tissue. These cells are especially numerous at sites of potential injury such as nose, mouth, feet, internal body surfaces, and blood vessels. Non-mast cell histamine is found in several tissues including the brain, where it functions as a neurotransmitter. Another important site of histamine storage and release is the enterochromaffin-like (ECL) cell of the stomach (Lee *et al.,* 2006). In response to an antigen, mast cells activation and degranulation are mediated by crosslinking of IgE which is bound to FcƐRI on mast cells. Through degranulation, histamine as well as a number of inflammatory cytokines are mediators releasing from mast cells. Among the inflammatory substances released from mast cells, histamine is the most potent vasoactive mediator and best-characterized mediator associated with the acute phase of hypersensitivity (Kim *et al*., 1999, Lee *et al*., 2006, and Shin *et al*., 2005). Histamine exerts its action from binding to histamine receptors on the target cells. Its main action includes gastric acid secretion, smooth muscle cells contraction, vasodilation and increased vasocular permeability (Choi *et al*., 2006).

Non anaphylactic mast cell activation can be mediated by compound 48/80, which stimulates trimeric G-proteins at mast cell membrane causing degranulation via phospholipase C and D pathways (Wang *et al*., 2005). Nevertheless, a high concentration of compound 48/80 is required in inducing almost 90% of histamine releasing from mast cells. An appropriate amount of compound 48/80 has been used in studying the mechanism of anaphylactic reaction (Kim *et al*., 1999).

To date, inhibitory effect on histamine release from mast cells is mostly examined by determination of in vitro released histamine. Determination of histamine is not straight forward because lacking of a chromophore in the molecule of histamine as well as and

very low levels in biological matrix (Arakawa *et al*., 1986, Douabale *et al*., 2003, and Previati *et al*., 2002). Many analytical procedures have been developed to quantitatively determine histamine release from mast cells (Jensen *et al.*, 1995). Previously reported, bioassay, RIA, GC, LC/MS methods are often encountered with either sensitivity problems, or high skilled operators requirement. The most successful methods always utilize precolumn derivatization prior to HPLC and fluorescence detection. This method has been the most commonly used because of its high resolution and high sensitivity (Solomon *et al*., 2002). 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was used as a derivatizing agent in the determination of histamine in red and white wine, which histamine was determined in μg/ml range (Hernandez-Orte *et al*., 2006). Another study using *o*-phthalaldehyde (OPA) as a derivatizing agent was reported for determination of histamine in human whole blood and plasma in concentration as low as ng/ml (Arakawa *et al*., 1986).

Due to the side effects of common antihistaminic drugs, such as drowsiness, dry mouth, blurred vision, nausea/vomiting, searching for anti-allergic resources other than pharmaceutical agents are achieving enormous interests. It has been reported that ellagic acid (2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3,–cde][1]benzopyran-5,10-dione), found in many woody plants and in diverse fruits and nuts, possess a variety of biological activities including anti-inflammatory activity, anti-oxidant, anti-fibrosis and anti-cancer properties (Masamune *et* al., 2005). Inhibition of histamine release by herbal crude extract is generally examined by determination of histamine released from mast cells. Normally, compound 48/80 was used to stimulate and induce mast cells degranulation and releasing histamine in vitro (Kim *et al*., 1999, Moon *et al*., 2005). Nowadays, inhibitory effect on histamine release from mast cells by crude extract from Thai herbs has not been reported.

This study aims to develop a versatile analytical method that can quantitatively determine histamine released from mast cells. Additionally, application of the developed method to study inhibitory effect of ellagic acid rich extracts on histamine release which was also demonstrated (Masamune *et* al., 2005).

1.2 Objectives

The objectives of this study were as follows:

1.2.1 To develop and optimize a method to quantitative/determine histamine released from mast cells by high-performance liquid chromatographic technique

1.2.2 To validate the developed method

1.2.3 To examine inhibitory effects of the crude extract as a model of inhibitory effect study of Thai herbal crude extract

CHAPTER 2

REVIEW OF LITERATURE

2.1 What is allergy?

Allergy is a disorder of the immune system that is often called atopy (Kim *et al*., 2005). Allergic reaction is mediated by histamine released from mast cells, derived from CD34+ hematopoietic progenitor cells, generally do not circulate in the blood but are the constituents of every organ and tissue. They are very long lived and retain the ability to proliferate under certain conditions especially at sites susceptible to injury such as the nose, mouth, feet internal body surfaces and blood vessels (Kim *et al*., 2005, Lee *et al.,* 2006). Mast cells play a major role in immediate-type allergic reaction by releasing chemical mediators such as histamine, serotonin and arachidonic acid metabolites. Allergy is mediated by degranulation of mast cells that when exposed to allergens. Among the inflammatory substances released on the degranulation of mast cells, histamine is the best characterized and most potent vasoactive mediator implicated in the acute phase of immediate-type allergic reaction (Kim *et al*., 1999, Lee *et al.,* 2006, Shin *et al.,* 2000, and Shin *et al*., 2005). The important step in mast cell activation appears to be the crosslinking of IgE molecules, which causes the clustering of Fc receptors. Bridging of receptors can also form hydrophilic channels that allow calcium passing and induced increasing intracellular calcium level. Moreover, it has been established that IgE acts as an anchor for an antigen which helps in amplifying the signal transduction (Shin *et al*., 2005, Lee *et al.,* 2006, Kim *et al*., 1999, Metcalfe *et al*., 1997, Froese, 1980). Non-mast cell histamine is found in several tissues, including enterochromaffin-like (ECL) cell of the stomach and in the brain. Functioning as a brain neurotransmitter mediating numerous important neurophysiological functions, such as circadian rhythm, sleep, feeding, drinking, temperature regulation, cognition, motion sickness, cardiovascular control, pain, aggressive behavior and hormonal secretion. It was found that repeat exposure to an allergen crosslinking of the IgE molecules on mast cell surface clusters the Fc receptors. This clustering stimulates a signal transduction causing the mast cell degranulation

(Figure 2-1). It also found that at least two specific IgE molecules were required to induce skin reaction in human.

Allergy (type I or immediate type hypersensitivity) is characterized by excessive activation of mast cells and basophils by IgE. IgE is considered a key mediator in the pathogenesis of allergic diseases as well as asthma (Charles *et al*., 2006).

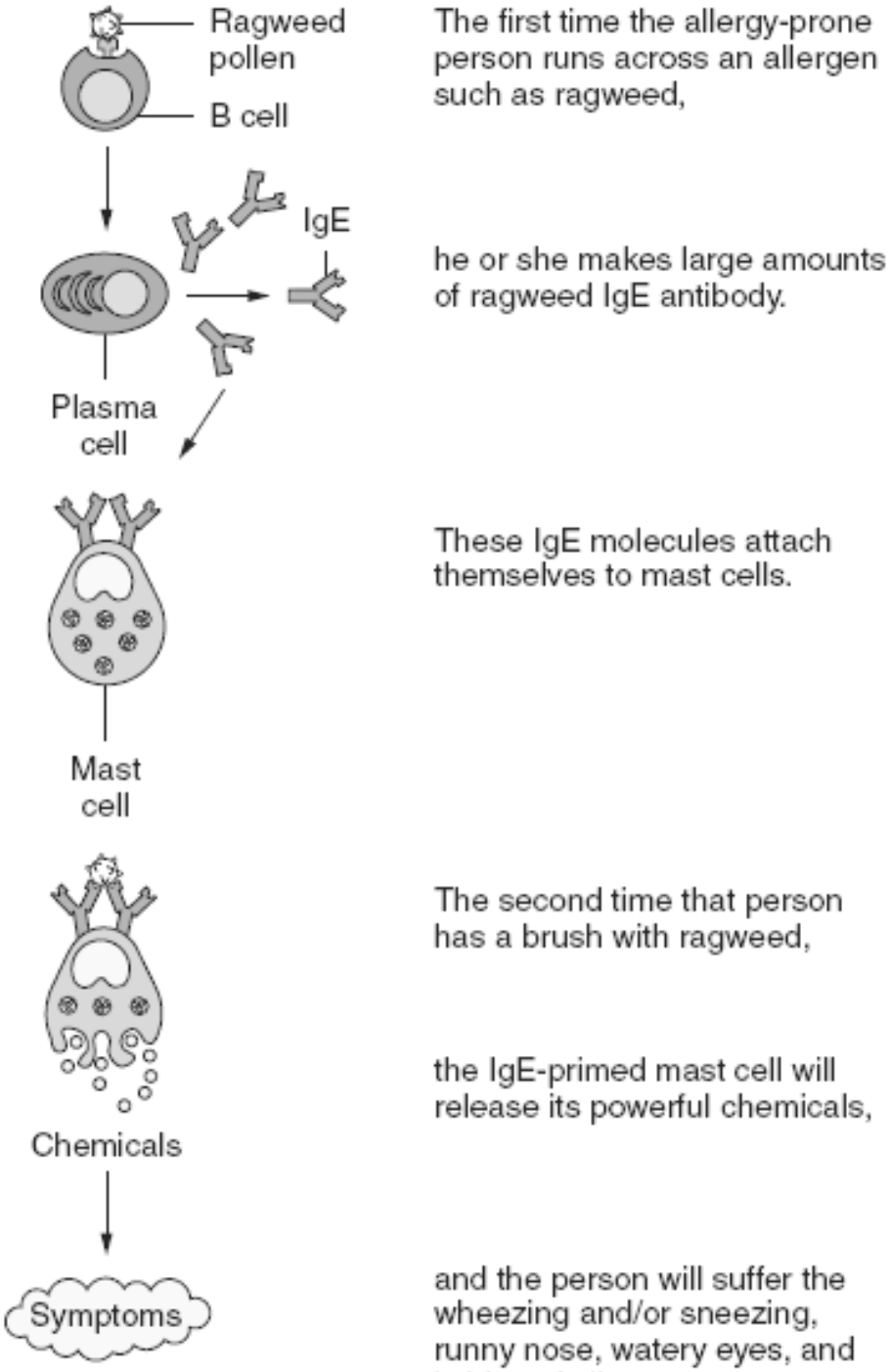
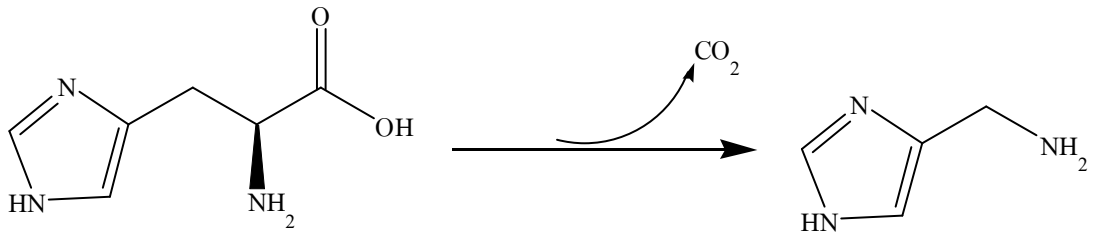


Figure 2-1 Mechanism for degranulation of mast cells (National Institute of Allergy and Infectious Diseases, USA)

2.2 Biology of Histamine

Histamine, 2-(4-imidazole)-ethylamine, a low MW biogenic amine, is an important chemical mediator in allergic reaction and inflammation (Matsubara *et al.,* 2005, and Tanimoto *et al*., 2006). Histamine is synthesized from L-histidine by histidine decarboxylase (HDC), an enzyme expressed throughout the body. including central nervous system neurons, gastric-mucosa parietal cells, mast cells, basophils, macrophage, and lymphocytes (Abe *et al*., 1994, Estelle *et al*., 2004 and Tanimoto *et al*., 2006).



Histidine Histamine

Figure 2-2 Decarboxylation of histidine

As shown in Figure 2-2, decarboxylation (Fig. 2-2) by HDC can be found in animal tissues and microorganisms (Douabale *et al*., 2003, Previati *et al.,* 2002). Mammalian HDC is a member of a large family of pyridoxal 5-phosphate (PLP)-dependent enzyme expressed in most tissue, but the highest levels are found in the skin, the gastrointestinal (GI) tracts, and the airways (August *et al*., 2006). Histamine was synthesized and stored mainly in mast cells and basophils where it is detained within granules (Bachert, *et al*., 2002, Matsubara, *et al*., 2005). These intracellular granules are complexed with an acidic protein, macroheparin and histamine dissociates from its complex by cation exchange with extracellular sodium at a natural pH. Histamine released into the synapses is broken down by acetylaldehyde dehydrogenase. It can also be degraded by histamine-*N*-methyltransferase and diamine oxidase (Choi *et al.,* 2006).

Mechanism of action

Addition to vasoactive action, histamine can cause the stimulation of gastric secretion, contraction of most smooth muscles, cardiac stimulation, vasodilation, and increased vascular permeability (Choi *et al.,* 2006).

Histamine exerts its action by binding to cellular histamine receptors. Four histamine receptors with different actions are shown in Table 2-1.

Table 2-1 Mechanism of action

Type	Location	Function
H_1	Smooth muscle, endothelium, and central nervous system tissue	Vasodilation, bronchoconstriction, smooth muscle activation, separation of endothelial cells (responsible of hives), and pain, and itching due to insect stings; the primary receptors involved in allergic rhinitis symptoms and motion sickness
H_2	Parietal cells	Primarily stimulate gastric acid secretion
H_3	-	Decreased neurotransmitter release: histamine, acetylcholine, norepinephrine, serotonin
H_4	Thymus, small intestine, spleen, and colon. It is also found on basophils and in the bone marrow.	Unknown physiological role

There are four major types of histamine receptor, H_1-, H_2-, H_3-, and H_4-receptor. These 4 types of histamine receptor differ in their expression, signal transduction, and function. H_1- in contrast to H_3- and H_4-receptors and H_2-receptors are more widely expressed. Histamine, which all are heptahelical that transduce extracellular signals by G proteins pathway. All have

constitutive activity, which is defined as the ability to rigger downstream events even in the absence of ligand binding. The active and inactive states of the receptors exist in equilibrium (Estelle *et al*., 2004).

Histamine has many inflammation effect and hypersensitivity, including vasodilation, edema, increased vascular permeability, and smooth muscle contraction (Rang *et al*., 1995). Increased vascular permeability causes fluid to escape from capillaries into the tissues, which leads to the classic symptom of an allergic reaction runny nose and watery eyes (Sompayrac, 1999). It is thought to be a major mediator of the acute inflammatory response, although histamine H1 antagonists have little effect on acute inflammation (Rang *et al*., 1995).

Gastric secretion: the most important action of histamine, in a clinical sense, is its stimulation of gastric acid secretion by acting on H2-receptors. It is implicated in the formation of peptic ulcers (Rang *et al*., 1995).

Smooth muscle Effects: histamine causes of the smooth muscle of the ileum, bronchi and bronchioles, and uterus by acting on H1-receptors. It may be involved in increased peristalsis associated with food allergies (Abbas *et al*., 1994). Histamine-induced bronchiolar has been implicated in the first phase of bronchial asthma (Rang *et al*., 1995). In asthmatics, histamine was found to increase airway smooth muscle tone and cause mucosal edema and glandular secretion, resulting in the narrowing of airways and limited air flow. In nonasthmatics, bronchial activity of histamine was limited, most likely due to fewer H1-receptors in airway smooth muscle (Goldie, 1990).

Cardiovascular effects: acting on H1-receptors, histamine causes the dilation of blood vessels; it induces endothelium cell to synthesize vascular smooth muscle relaxants, including prostacyclin and nitric oxide, which cause vasodilation (Abbas *et al*., 1994). Acting on H2-receptors, it increases heart rate and cardiac output. When injected intradermally, histamine leads to reddening of the skin, wheal, and flare, called the “triple response”. Vasodilation of small arterioles and precapillary sphincters causes reddening, while increased permeability of postcapillary venules

capillary permeability. Histamine also induces an “axon reflex”, which leads to stimulation of sensory nerve fibers and the release of a vasodilator mediator; this the flare (Rang *et al*., 1995).

Itching: if histamine is injected into the skin, it causes itching, due to stimulation of sensory nerve ending (Rang *et al*., 1995).

Allergens can bind to IgE-loaded mast cells in the nasal mucosa, which leads to three clinical responses: sneezing which is results from histamine-associated sensory neural stimulation; hypersecretion from glandular tissue occurs; nasal mucosal congestion due to vascular engorgement caused by vasodilation and increased capillary permeability (Monroe *et al.,* 1997).

2.3 Analytical method for histamine determination

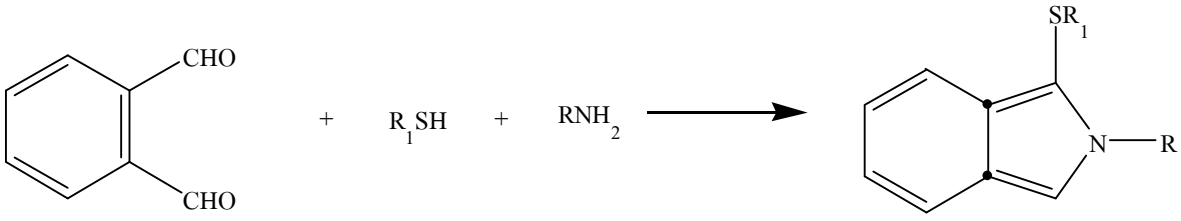
Histamine has been shown to play important roles in allergy, microcirculation, and gastric acid secretion, neurotransmitter, and to participate in some adverse reaction to drugs. Histamine in tissues and body fluids is important in various physiological investigations (Arakawa *et al*., 1986). A good starting point in understanding the process that leads to allergic symptom is the mast cell. The mast cell remains at the core of understanding of allergic inflammation and is thus a major target for future therapeutic interventions in allergy (Saito *et al*., 2005).

Mast cell degranulation can be elicited by a number of positively charged substances, known as the “basic secretagogues” of mast cells, such as compound 48/80 or NaF (Na *et al*., 2002, and Shin *et al.,* 2000). The synthetic compound 48/80 is known to be one of the most potent secretagogues of mast cells. Studies on the compound 48/80-induced mast cell degranulation and ear-swelling response have been continuously performed on a theoretical basis by Kim et al. (Na *et al*., 2002). Compound 48/80 is a mixture of polymers synthesized by condensing *N*-methyl-*p*-methoxyphenyl ethylamine with formaldehyde, and its hypotensive effect, resulting from histamine release. Compared with the natural process, a high concentration of compound 48/80 induces almost a 90% release of histamine from mast cells. Thus, an

appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylactic allergy reaction (Na *et al*., 2002, and Shin *et al.,* 2000). The secretory response of mast cells can be induced by aggregation of their cell surface-specific receptors for IgE by the corresponding antigen (Moon *et al*., 2005, and Na *et al*., 2002). It has been established that the anti-IgE antibody induces passive cutaneous anaphylaxis (PCA) as a typical model for mast cell-dependent immediate type allergic reaction (Na *et al*., 2002).

Since its discovery in 1916 by Gugenheim and Lofler, a variety of techniques and detectors have already been used for determining histamine in various matrices (Douabale *et al*., 2003). Analytical determination of histamine is not simple because of its structure and it is usually present at low levels in a biological matrix (Arakawa *et al*., 1986, Douabale *et al*., 2003, and Previati *et al*., 2002). Many types of assay methods can be used; the bioassay, the radioenzymatic assay, and fluorometric assay. The biological assay is not sensitive or selective enough to directly determine histamine in biological matrices unless additional extraction and purification steps are taken (Arakawa *et al*., 1986). The originally developed methods are based on enzymatic or radio enzymatic assays. The radioenzymatic assay using histamine methyltransferase and ^{14}C or *S*-[^{3}H] adenosylmethionine (SAMe) has been improved so that it is now a highly sensitive method. However, it requires the use of a highly purified enzyme and commercially unavailable, the complete separation of the isotope-labeled product from ^{14}C or [^{3}H] SAMe after incubation, and special facilities for the handling of radioisotopes, which are not suitable for routine assay in normal laboratories (Arakawa *et al*., 1986, and Douabale *et al*., 2003). The most successful assays rely on precolumn derivatization followed by high-performance liquid chromatography (HPLC) and fluorescence detection. This method has been the most commonly used because of its high resolution, sensitivity and selectivity of quantifying low levels of histamine in biological matrices. Employment of the use of fluorescence for histamine analysis developed by Shore et al. in 1959, and have been widely used (Arakawa *et al*., 1986, Douabale *et al*., 2003, Previati *et al*., 2002, and Solomon *et al*., 2002). Histamine has no chromophore or electrophore, making detection a challenge. Among available fluorescence labeling reagents, dansyl chloride (DnsCl), *o*-phthalaldehyde (OPA), 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), were used either pre-column, on-column or post-column derivatization, for HPLC analysis with fluorescent detection. The most important advantage

offered by OPA is that it reacts with primary amines quickly enabling the biogenic amine detected at fmol levels (Figure 2-3)(Busto *et al*., 1997).



(OPA) (2-mercaptoethanol) (primary amine) (alkylthiolisoindoles)

Figure 2-3 OPA derivatization of a primary amine in the presence of a thiol

OPA rapidly reacts with primary amines to form alkylthiolisoindoles, which are electrochemically active, fluorescent, and UV-absorbing. Moreover, combination of OPA, an appropriate thiol, and pH control, a rapid derivatization and formation of a stable product can be obtained (Solomon, 2002).

Previously studied HPLC conditions for determination of histamine are listed in Table 2-2.

Table 2-2 HPLC conditions for determination of histamine

Samples	**Stationary phase**	**Mobile phase**	**Flow rate (ml/min)**	**Derivatization**	**Detection**	**Ref.**
Human mast cells line	A Delta-Pak HPI C4 300 A°, 2.0 × 150 mm column (Waters, Milford, MA)	20 ml PIC® B-8 Low UV Reagent (Waters) in 1 l of 10 mM triethylamine phosphate, pH 3.0	0.2	-	UV at 215 nm	August *et al*., 2006

Samples	Stationary phase	Mobile phase	Flow rate (ml/min)	Derivatization	Detection	Ref.
Red wines	Asahipack OP-50 cartridge supplied by Hewlwtt-Packard	Gradient elution; eluent (A): 5 mM borate buffer (pH9)/1%THF, (B): 12 mM OPA-NAC/5 mM borate (pH 9), (C): ACN	0.8	OPA	Fluorescence detection at λ_{ex}=340 nm and λ_{em}=450 nm	Busto *et al*., 1997
Tuna fish	Spherisorb ODS2 C18, 25 × 4.6 mm (5 μm) column (Alltech, Milan, Italy)	20 mM KH_2PO_4, pH 3.0	0.5	-	UV detection at λ=215 nm	Frattini *et al*., 1998
Tuna fish	Spherisorb ODS2 C18, 25 × 4.6 mm (5 μm) column (Alltech, Milan, Italy)	20 mM KH_2PO_4 (pH 4.0) : acetonitrile (7:3)	0.8	OPA	Fluorescence detection at λ_{ex}=315 nm and λ_{em}=415 nm	Frattini *et al*., 1998
Whole blood of colon cancer patients	C18 Ultrasphere column (Beckman, Milan, Italy)	50 mM acetate buffer, pH 4.0:methanol:acetonitrle (44:20:36) containing 8 mM sodium decanesulfonate (DSF)	1.0	OPA	Fluorescence detection at λ_{ex}=360 nm and λ_{em}=450 nm	Previati *et al*., 2002

Samples	Stationary phase	Mobile phase	Flow rate (ml/min)	Derivatization	Detection	Ref.
Standard solution of histamine	Hypersil ODS	Gradient elution. Eluent A: 0.05 M sodium acetate (pH 7.2). Eluent B: 0.1 M sodium acetate-acetonitrile-methanol(46:44:10) (pH 7.2)	1.0	OPA	Fluorescence detection at λ_{ex}=337 nm and λ_{em}=454 nm	Kutlan *et al*., 2002
Mast cells of mouse	C18 spherisorb ODS column (Beckman)	Methanol:0.02 M sodiumacetate (1:1, v/v) supplemented with 5 mM 1-octanesulfonic acid	1.0	OPA	Fluorescence detection at λ_{ex}=360 nm and λ_{em}=455 nm	Egger *et al*., 1994
Wines	Waters Nova-Pak C18	Gradient elution. Eluent A: $Na_2HPO_4 \cdot 12H_2O$ (3.6 mg/l, 10 mM). Eluent B: 1% 2-octanol in acetonitrile and eluent A (70:30 v/v)	0.8	OPA	Fluorescence detection at λ_{ex}=340 nm and λ_{em}=425 nm	Marcobal *et al*. 2005
Mast cells	Ace5 C18 with a Zorbax C18 guard column	0.1 M H_2NaPO_4 and 1 mM Na_2EDTA with 16% methanol and 14%acetonitrile, pH 6.4	0.8	OPA	Fluorescence detection at λ_{ex}=348 nm and λ_{em}=444 nm	Gordon *et al*., 2004

Samples	Stationary phase	Mobile phase	Flow rate (ml/min)	Derivatization	Detection	Ref.
Meat products	Zorbax Eclipse XDB C8 column (150 mm×4.6 mm, particle size 5 µm) with guard column Meta Guard Inertsil C18 (30 mm×4.6 mm, particle size 5 µm)	Gradient elution: 100 mM acetate buffer (pH 5.8) and acetonitrile	0.6	OPA	Fluorescence detection at λ_{ex}=330nm and λ_{em}=440 nm	Smela *et al*. 2003
Epithelia of pig colon	EC 125/4 Reprosil-Pur C18 AQ (Trentec-Analysentechnik, Germany)	30 mM $Na_2Ba_4O_7$ in methanol-water (40/60; v/v) and 0.2 mM OPA	1.5	OPA	Fluorescence detection at λ_{ex}=350 nm and λ_{em}=450 nm	Vietinghoff *et al*., 2006
Meat products	Zorbax Eclipse XDB C18 column (150×4.6 mm, partial size 5 µm) with guard column Meta Guard ODS-2 (30 mm×4.6 mm)	Gradient elution: H_2O and acetonitrile	0.8	Dansylchloride	UV/VIS detector at 254 nm	Smela *et al*. 2003

Samples	Stationary phase	Mobile phase	Flow rate (ml/min)	Derivatization	Detection	Ref.
Chocolate	Dionex CS17	Gradient elution aqueous methanesulfonic acid	0.35	-	Electrochemical detector	Onal. 2007
Meat products	IonPac CS17 column, a cation-exchange column	Gradient elution methanesulfonic acid	1.0	-	UV and MS	Onal. 2007
Wines	Luna C18	Gradient elution. Eluent A: buffer, pH8 (30 ml)+acetonitrile (550 ml)+H_2O (420 ml). Eluent B: buffer, pH 8 (2 ml)+acetonitile(900 ml)+H_2O(100 ml)	-	OPA	Diode array detector (DAD): 200-550 nm	Onal. 2007
Tuna fish	Luna C18	Gradient elution. Eluent A: 85% of buffer solution (pH 6.9) and 15% of methanol. Eluent B: acetonitrile	-	-	DAD	Cinquina *et al.* 2004

Samples	Stationary phase	Mobile phase	Flow rate (ml/min)	Derivatization	Detection	Ref.
Musts and wines	Luna C18 bonded silica (Torrance, USA)	Gradient elution. Eluent A: 140 mM sodium acetate trihydrate and 17 mM TEA (pH 5.05), Eluent B: methanol	1.0	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate	Fluorescence detection at λ_{ex}=250 nm and λ_{em}=395 nm	Hernandez-Orte *et al*., 2006
Alcoholic beverages	Nova-Pak C18	Gradient elution. Eluent A: 0.1 M sodium acetate and 10 mM sodium octanesulfonate (pH 5.3). Eluent B: mixture of solvent B-acetonitrile (6.6:3.4), where solvent B was a solution of 0.2 M sodium acetate and 10 mM sodium octanesulfonate solution adjusted to pH 4.5	1.0	Post-column derivatizing with OPA	Fluorescence detection at λ_{ex}=340nm and λ_{em}=445 nm	Vidal-Carou *et al*. 2003
Alcohol beverages	Inertsil ODS-3	Gradient elution (A) H_2O and (B) acetonitrile	1.0	Dansylchloride	Fluorescence detection at λ_{ex}=320nm and λ_{em}=523 nm	Loukou *et al*. 2003

Samples	Stationary phase	Mobile phase	Flow rate (ml/min)	Derivatization	Detection	Ref.
Brain microdialy-sis samples from awake rats	Microbore colum paked with C18 silica gel (Tokyo, Japan)	A mixture containing 75% acetonitrile in water	50 µl/min	4-(1-pyrene)butyric acid *N*-hydroxysuccini mide ester (PSE)	Fluorescence detection at λ_{ex}=345 nm and λ_{em}=500 nm	Yoshitak e *et al*., 2003
Coffee	Kromasil C18	Gradient elution; 0.5 mM phosphoric: acetonitrile : methanol	1.0	Dansylchloride	Diode array detector at 254 nm, connected in series with a fluorimetric detection at λ_{ex}=252 nm and λ_{em}=500 nm	Casal *et al*. 2002
Fish and fishery products	C18-µ-Bondapak	Gradient elution; methanol: H_2O	1.0	-	UV detector at 254 nm	Shakila *et al*. 2001
Fermented cabbage juices, soy sauces	Grom-Sil a ODS-3 CP 120 RP-18 encapsulated polymer coated column	Gradient elution. Eluent A: 100 mM NaOAc, pH 7.0; eluent B: 100 mM NaOAc, pH 4.3; eluent C: acetonitrile	-	3,5-dinitrobenzoyl chloride (DNBZ-Cl)	UV-absorption at 260 nm	Kirschba um *et al*. 2000

Samples	Stationary phase	Mobile phase	Flow rate (ml/min)	Derivatization	Detection	Ref.
Wines	LiChrospher 100 RP-18	Gradient elution. Elutent A, consisting of sodium acetate, 10%(v/v) dimethylformamide, and 0.23%(v/v) triethylamine (pH 5). Eluent B, consisted of acetonitrile-tert.-butylmethylether-water butylmethylether-water (87.5:10:2.5, v/v/v).	1.0	Dansyl chloride	Spectrophotometric detection at 446 nm	Romero *et al*. 2000
Wines	Asahipack OP-50 cartridge	Gradient elution (A) 5 mM borate solution (pH 9) with 1% THF, (B) 5 mM borate solution (pH 9) with 12 mM OPA-NAC, and (C) CAN	0.8	OPA	Fluorescence detection at λ_{ex}=340nm and λ_{em}=450 nm	Busto *et al*. 1997
Fish tissues	IonPac CS17 column	Gradient elution: methanesulfonic acid gradient	1.0	-	Electrochemical detection	Cinequina *et al*. 2004

Samples	Stationary phase	Mobile phase	Flow rate (ml/min)	Derivatization	Detection	Ref.
Wines	Synergi Hydro-RP C18	Gradient elution. Eluent A: 2%(v/v) acetic acid aqueous solution, Eluent B: methanol as organic modifier	1.0	1,2-naphthoquinone-4-sulfonate	Spectrophotometric detection at 305 nm and 270 nm	Onal. 2007
Spinach, hazelnut, banana, potao, and milk chocolate	Nova-Pak C18	Gradient elution. Eluent A: a solution of 0.1 M sodium acetate and 10 mM sodium actanesulfonate (pH5.23), eluent B: mixture of solvent B-acetonitrile (6.6:3.4), where solvent B was a solution of 0.2 M sodium acetate and10 mM sodium octanesulfonate solution adjusted to pH 4.5 with acetic acid	1.2	OPA	Fluorescence detection at λ_{ex}=340nm and λ_{em}=445 nm	Lavizzari *et al*., 2006

Samples	Stationary phase	Mobile phase	Flow rate (ml/min)	Derivatization	Detection	Ref.
Wines	A Zorbax C8 column	Gradient elution. Eluent A: 15% CAN + 85% aqueous solution (15 mM sodium heptanesulfonate (SHS) + 10 mM phosphoric acid); eluent B: 70% ACN + 30% aqueous solution (8 mM SHS + 10 mM phosphoric acid)	0.8	1,2-naphthoquinone-4-sulfonate	Spectrophotometric detection at 305 nm	Hlabangana *et al*. 2006
Wines	Inertsil ODS-2 and 3	Gradient elution; acetonitrile:water	1.0	Dansyl chloride	UV detection at 254 nm	Soufleros *et al*. 2007
Cheese	Romasil KR 100-5 C18	Gradient elution; acetonitrile:water	0.8	-	Spectrophotometric detection at 254 nm	Innocente *et al*., 2006

2.4 Study on the inhibition of histamine release by herbal plant

In general, immediate allergic reaction, which includes urticaria, allergic rhinitis, and asthma, is mediated by various chemical mediators released from mast cells. Mast cell degranulation can also be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells. Compound 48/80 is one of the most potent secretagogues of mast cells. On a theoretical basis, studies on compound 48/80-induced mast cell

degranulation and ear swelling response have been examined by Kim et al. (Hong *et al*., 2001, Kim et al., 1999). The secretory response of mast cells can be induced by aggregation of their cell surface-specific receptors for IgE by the corresponding antigen. It has been established that the anti-IgE antibody induces passive cutaneous anaphylaxis (PCA) as a typical model for mast cell-dependent immediate type allergic reaction (Choi *et al*., 2006, Hong *et al*., 2001, Kim *et al*., 1999, Kim *et al*., 2005).

Gahmi-Shini-San (GSS) is an orient herbal medication that has been used to threat anaphylactic skin diseases in Korea. GSS showed inhibition for the ear swelling response induced by intradermal injection of compound 48/80 in a mouse model on a concentration-dependent basis. GSS significantly inhibited the compound 48/80-induced degranulation from mast cells in ear tissue, and GSS dose-dependently inhibited the histamine release from the rat peritoneal mast cells by compound 48/80. GSS showed inhibition of passive cutaneous anaphylaxis following oral administration that studied the effect of GSS on mast cell-dependent passive cutaneous anaphylaxis activated by dinitrophenyl IgE antibody. These results indicated that GSS has inhibitory effect on mast cell-dependent immediate type cutaneous reaction (Hong *et al*., 2001).

The gall of *Rhus javanica* (GRJ) is the excrescence produced by parasitic aphids, mainly *Melaphis chinensis* (Bell) Baker, on the leaf of *Rhus javanica* L. (Anacardiaceae). This crude drug has the pharmacological functions of antibacterial activity, antivirus activity and antihelicobacter pylori activity, and has been used for chronic cough, hematochezia, abscess, skin disease and diabetes. GRJ inhibited compound 48/80-induced systemic reactions in mice. GRJ attenuated IgE-mediated local allergic reactions, and dose dependently decreased histamine release from rat peritoneal mast cells activated by compound 48/80 (Kim et al., 2005).

Cichorium intybus (Compositae) is commonly known as ‘Chicoryo’. An aqueous extract of *Cichorium intybus* (CIAE) dose-dependently inhibited systemic anaphylactic reaction induced by compound 48/80 in mice. Especially, CIAE inhibited compound 48/80-induced anaphylactic reaction 100% with the dose of 1000 mg. The plasma histamine levels in mice were reduced in a dose-dependent manner, when they were pretreated with CIAE at a concentration ranging from 0.1 to 1000 mg/kg. CIAE (1-1000 μg/ml) dose-dependently inhibited histamine

release from the rat peritoneal mast cells activated by compound 48/80 or anti-DNP IgE (Kim *et al*., 1999).

Phellinus linteus, an orange color mushroom growing well on mulberry tree, is a well-known fungus of the genus *Phellinus* in the family of Hymenochaetaceae and has been used as a traditional herb medicine in oriental countries and is known to have anti-tumor, immunomodulatory, anti-inflammatory, and anti-allergic activities. Oral administration of water extract from the fruiting body of *Phellinus linteus* (WEPL) inhibited the compound 48/80-induced systemic anaphylaxis-like reaction and ear swelling response. WEPL also inhibited the anti-dinitrophenyl (DNP) IgE-mediated passive systemic and cutaneous anaphylaxis. WEPL dose-dependently reduced histamine release from rat peritoneal mast cells activated by compound 48/80 or anti-DNP IgE (Choi *et al*., 2006).

Agaricus blazei is a medicinal mushroom native to Brazil. It used to be a source of anti-tumor and immunoactive compounds and considered a health food in many countries. *Agaricus blazei* water extract (ABWE) inhibited compound 48/80-induced systemic anaphylaxis-like reaction, ear-swelling response, passive cutaneous anaphylaxis-like reaction in mice. ABWE also inhibited the anti-dinitrophenyl (DNP) IgE-mediated passive systemic and cutaneous anaphylaxis. ABWE dose-dependently reduced histamine release from rat peritoneal mast cells activated by compound 48/80 or anti-DNP IgE (Choi *et al*., 2006)

The fruits of *V. rotundifolia* (L.) (Verbenaceae) (VRFE), well known as ‘Man Hyung Ja’ in Korea, have been used against headache in upper respiratory infection and they still occupy an important place in traditional Oriental medicine for treatment of various allergic diseases through various administration routes in Korea. VRFE dose-dependently inhibited systemic allergic reaction induced by compound 48/80 and inhibitd passive cutaneous anaphylaxis activated by anti-dinitrophenyl (DNP) IgE. VRFE dose-dependently reduced histamine release from rat peritoneal mast cells activated by compound 48/80 or anti-DNP IgE (Shin *et al*., 2000).

Buckwheat grain extract (BGE) is one of the widely consumed foodstuffs. BGE has long been used as therapeutics for anti-inflammation, detoxification and lowering the fever in Korean folk medicine. BGE showed potent inhibitory effect on passive cutaneous anaphylaxis (PCA) activated by anti-dinitrophenyl (DNP) IgE when orally administered and inhibitory

potential on the compound 48/80-induced histamine release from rat peritoneal mast cells (RPMC) (Kim *et al*., 2003).

Tongkytang (TKT) is an Oriental herbal prescription, which has been successfully applied for the treatment of allergic disorders, mainly allergic-rhinitis in clinical medicine. TKT concentration-dependently inhibited the ear-swelling response induced by intradermal injection of compound 48/80. TKT inhibited the compound 48/80-induced degranulation from mast cells in ear tissue. TKT dose-dependently inhibited the histamine release from the rat peritoneal mast cells by compound 48/80 (Na *et al*., 2002).

Gamibojungikgitang (GBIT) is an Oriental herbal prescription medication, which has been commonly used to treat allergic rhinitis in far Eastern countries including Korea, China, and Japan. GBIT extract dose-dependently inhibited compound 48/80-induced systemic anaphylactic shock. GBIT dose-dependently inhibited the histamine release from the rat peritoneal mast cells by compound 48/80. GBIT showed potent inhibitory effect on passive cataneous anaphylaxis (PCA) activated by anti-dinitrophenyl (DNP) IgE (Moon *et al*., 2005).

Xanthii fructus (XF) which is well known as “Chang-ihjah” in Korea is the dired fruit of *Xanthium strumarium* L. Water extract of this fruit has been used for treatment of various inflammatory diseases such as tympanitis, allergic rhinitis, or ozena as an alternative therapy material usually by oral administration in far Eastern countries including Korea. The effect on mast cell-mediated allergic reaction, XF inhibited compound 48/80-induced systemic anaphylaxis in mouse. This dose-dependently inhibited histamine release from rat peritoneal mast cells by compound 48/80 (Hong *et al*., 2003).

In this study, ellagic acid rich extracts from *Punica granatum* was examined for histamine release inhibitory effect study. Ellagic acid (2,3,7,8-tetrahydroxy[1] benzopyrano[5,4,3,–cde][1] benzopyran-5,10-dione)(Figure 2-2), is a hydrolytic chemical found in *Punica granatum,* ellagic acid derivatives product from ellagic acid glycosides and ellagitannins (Lee, 2004). Ellagic acid has a variety of biological activities including anti-oxidant, anti-inflammatory, anti-fibrosis and anti-cancer properties in bacterial and mammalian systems.

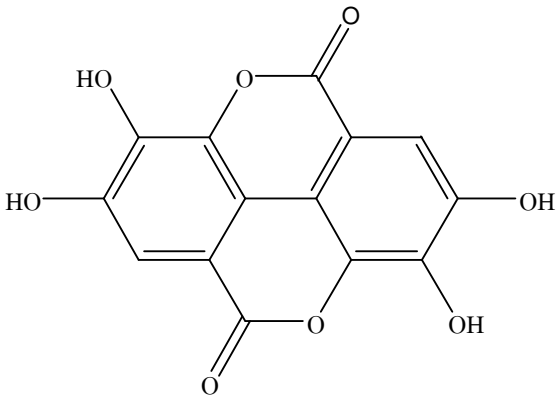


Figure 2-4 Chemical structures of ellagic acid

Also, application of the developed method to study inhibitory effect by ellagic acid rich extracts was also demonstrated as a model for the study of inhibitory effect by various Thai herbal crude extracts.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Materials and Equipments

3.1.1 Materials

Acetonitrile and triethylamine used in this study were HPLC grade obtained from Lab-Scan, Analytical Sciences, Thailand. The other chemicals were analytical grade. All chemicals and solvents are listed below.

Chemicals	**Source**
Boric acid	Fluka, Switzerland
BSA (Albumin from bovine serum, lyophilized)	Fluka, Switzerland
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	AnalaR®, England
Dimethylsulphoxide $(CH_3)_2SO$	Lab-Scan, Analytical Sciences, Thailand
di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	Fluka, Germany
D-(+)-Glucose, anhydrous	Ajax Finechem, Australia
Ethanol	Siribuncha Co., LTD, Thailand
Ethylenediaminetetraacetic acid (Na_2EDTA)	Aiax Finechem, Australia
Gelatin	Fluka, Switzerland
Heparin Sodium salt from bovine intestinal mucosa	Biochemika, Fluka, Germany
HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulonic acid] ($C_8H_{18}N_2O_4S$)	Fluka BioChemika, USA
Histamine dihydrochloride	Fluka, Biochemika, Japan
Ketotifen fumarate salt ($C_{19}H_{19}NOS \cdot C_4H_4O_4$)	Sigma, Japan

L-histidine ($C_6H_9N_3O_2$)	Sigma-Aldrich, Germany
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	AnalaR®, England
2-mercaptoethanol (2-ME)(C_2H_6OS)	Sigma-Aldrich, Germany
Methanol (CH_3OH)	Lab-Scan, Analytical Sciences, Thailand
o-phthaldialdehyde (OPA)($C_8H_6O_2$)	Sigma, Germany
Percoll®	Fluka BioChemika, Sweden
Potassium chloride (KCl)	Ajax Finechem, Australia
Sodium chloride (NaCl)	AnalaR®, England
Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$)	Merck, Germany
Sodium hydroxide	Lab-Scan, Analytical Sciences, Thailand
Trypan blue	Invitrogen Corporation, USA
Ellagic acid rich extraced from *Punica granatum* peels	Assoc. Prof. Dr. Pharkphoom Panichayupakaranant

3.1.2 Equipments

High Performance Liquid Chromatography (HPLC) system: Shimadzu, Japan

- System controller: SCL-10AVP model
- Pump: LC-10ADVP (Liquid Chromatograph) model
- Degasser: DGU-14A model
- Automatically Injector: SIL-10ADVP model
- Fluorescence Detector: RF-10AXL model
- Column oven: CTO-10ASVP model

HPLC column: ODS-C18 column (Sunfire™, Water), 0.46 x 15 cm, 5 μm, and security guard column (Phenomenex®, USA) was packed with C18 particles, 4×3 mm

Sonicator:	Ultrasonicator, Chest
Centrifuge:	Hermle Labortechnik (Z 323K), Germany
Micro pipette:	RAININ, USA
Vortex:	Scientific Industries, INC., USA
Vial insert:	Agilent, USA
Precision pipette tips:	RAININ, USA
Microtubes:	Axygen® Scientific, USA
Hand tally counter:	SDI corporation, Taiwan
Neubauer Improved Bright-Line:	0.0025 mm^2 , 0.100 mm Tiefe Depth Profoudeur, Precicolor HBG, Germany
Deckglaser for Haemocytometer: (cover glass)	20×26 mm In vitro diagnostic, Menzel-Glaser®
Terumo® Syringe:	20 cc, Terumo®, Philippines

3.2 Methods

3.2.1 Preparation of standard solutions

Standard stock solution of histamine was prepared by accurately weighting standand histamine dihydrochloride 10 mg and dissolving in 50% methanol (in DI water) to yield a final volume of 10 ml (final concentration 1 mg/ml). This stock solution was used in preparing working histamine standard solution for routine analyses. The stock solution was stable for 1 month at -4^{o}C in refrigerator and kept in dark. Working solution of standard histamine was subsequently prepared in MT-HEPES-BSA buffer, pH 7.4 and diluted to provide series solution of histamine for use in constructing calibration curves for each of analysis.

3.2.2 Preparation of mobile phase

Mobile phase consists of mixture of aqueous phase and acetonitrile and methanol. The aqueous phase consists of 0.1 M NaH_2PO_4 with 1 mM EDTA and 0.1%(v/v) TEA.

Acetonitrile and methanol were added and adjusted to a final pH 6.4 with 1 M NaOH. The ratio of the mobile phases was prepared by mixing phosphate buffer (obtained %v/v TEA) : acetonitrile : methanol in the proportions as shown in Table 3-4 for finding an optimal separation. Mobile phases were filtered and sonicated before use.

3.2.3 Preparation of derivatizing agent

Derivatizing agent was 1:1 mixture of 0.1%(w/v in methanol) *o*-phthalaldehyde and 2.5 ml/l (in methanol) of 2-mercaptoethanol. This solution was kept in the dark and used within a day.

3.2.4 Preparation of Modified Tyrode-HEPES-BSA buffer, pH 7.4

Modified Tyrode-HEPES-BSA buffer solution was prepared using chemical listed in Table 3-1 and adjusted to pH 7.4 with 1 M NaOH. This solution was steriled by sterile filtration using 0.22 μm membrane filter, and kept in refrigerator.

Table 3-1 Chemicals used in preparation of Modified Tyrode-HEPES-BSA buffer solution (250 ml)

Item	Reagents	Concentration	MW	g/250 ml
1	Sodium chloride	137 mmol/l	58.44	2.0013
2	Potassium chloride	2.7 mmol/l	74.56	0.0502
3	Calcium chloride, dihydrate	1.8 mmol/l	147.02	0.0662
4	Magnesium chloride, hexahydrate	1 mmol/l	203.3	0.0508
5	Sodium dihydrogen phosphate, dihydrate	0.4 mmol/l	156	0.0156
6	D-(+)-Glucose, anhydrous	5.6 mmol/l	180.16	0.2522
7	HEPES	20 mmol/l	238.31	1.1915
8	Gelatin	0.05%		0.125
9	BSA	0.1%		0.25

3.2.5 Preparation of 60%Percoll® solution

90%Percoll® was 9:1 mixture of 100% Percoll® and 10xKrebs Ringer Phosphate (KRP) buffer solution. This solution was diluted to give 60% Percoll® with 1xKRP solution.

3.2.5.1 Preparation of 10 x KRP buffer, pH 7.4 [Ca(-), Glc(-), Mg(+)]

10 x KRP buffer solution was prepared using chemicals listed in Table 3-2 and adjusted to pH 7.4 with 1 M NaOH**.** This solution was steriled by steriled filtration using 0.22 μm membrane filter, and kept in refrigerator.

Table 3-2 List of chemicals used in preparation of 10 x KRP buffer solution (50 ml)

Item	Reagents	Concentration	MW	g/50 ml
1	Sodium chloride	9.00%	58.44	4.5
2	Potassium chloride	60 mmol/l	74.56	0.2237
3	Magnesium chloride, hexahydrate	10 mmol/l	203.3	0.1017
4	Sodium dihydrogen phosphate, dihydrate	15.0 mmol/l	156	0.117
5	Disodium hydrogen phosphate, dodecahydrate	85.0 mmol/l	358.14	1.5221

3.2.5.2 Preparation of 1 x KRP buffer, pH 7.4

1 x KRP buffer solution was prepared using chemicals listed in Table 3-3 and adjusted to pH 7.4 with 1 M NaOH**.** This solution was steriled by sterile filtration using 0.22 μm membrane filter, and kept in refrigerator.

Table 3-3 List of chemicals used in preparation of 1 x KRP buffer solution (250 ml)

Item	Reagents	Concentration	MW	g/250 ml
1	Sodium chloride	0.90%	58.44	2.25
2	Potassium chloride	6 mmol/l	74.56	0.1118
3	Magnesium chloride, hexahydrate	1 mmol/l	203.3	0.0508
4	Sodium dihydrogen phosphate, dihydrate	1.50 mmol/l	156	0.0585
5	Disodium hydrogen phosphate, dodecahydrate	8.50 mmol/l	358.14	0.7611
6	D-(+)-Glucose, anhydrous	10 mmol/l	180.16	0.4504

3.2.6 Preparation of rat peritoneal mast cells (RPMC)

Wistar rats were sacrificed by neck dislocation. The abdominal surface was wiped with 50% ethanol. Twenty ml of MT-HEPES-BSA buffer containing 5 units/ml of freshly prepared heparin was injected into peritoneal cavity. The abdomen was gently massaged for approximately 90 s before the peritoneal cavity was carefully opened. Peritoneal fluid (containing cellular components) was subsequently aspirated into a polypropylene tube using a pasteur pipette. Thereafter, the peritoneal fluid were centrifuged at 1,100 rpm, 5^{o}C for 5 min, the supernatant fluid was discarded. Cell pellets were washed twice with 10 ml MT-HEPES-BSA buffer and centrifuged at 1,100 rpm, 5^{o}C for 5 min. Cell pellets were reconstituted with 2 ml of MT-HEPES-BSA buffer yielding purified mast cells suspension. Aliquots of the cell suspension were pooled for mast cell separation.

Mast cells were separated from the major components of rat peritoneal cells, (i.e. macrophages and small lymphocytes) by density centrifugation with 60% Percoll® solution according to the following procedure. Cells suspension 1 ml were layered on 6 ml of 60% Percoll® solution and centrifuged at 1,700 rpm, for 15 min at room temperature. Discard the cells remaining at the MT-HEPES-BSA buffer layer, and 60% Percoll® layer. Cell pellets were reconstituted with 5 ml of MT-HEPES-BSA, and centrifuged at 1,700 rpm, for 15 min at room temperature. Centrifugation was repeatedly performed and cell pellets were reconstituted with 0.5 ml of MT-HEPES-BSA solution, and pooled for cell counts.

3.2.7 Counting of viable mast cells

Viability of the cells was examined by 0.4% trypan blue uptake. Cell suspensions were dyed with 0.4% trypan blue solution in 1:1 ratio, then performed cell count using hemocytometer. About 2.8×10^6 cells/ml of viable cells was used for study the inhibitory of histamine released from mast cells by ellagic acid rich extracts from *Punica granatum*.

3.2.8 Development of quantitative analysis of histamine in MT-HEPES-BSA Buffer, pH 7.4

3.2.8.1 HPLC system for assay development

Column:	ODS, C18 column (Sunfire, Water®), 0.46 x 15 cm, 5 μm, and guard column was packed with C18 particles	
Flow rate:	1.0 ml/min	
Volume of injection:	30 μl	
Detector:	Fluorescence;	excitation 348 nm emission 444 nm

3.2.8.2 Optimization of mobile phase of HPLC condition for analysis of histamine (modified from Gordon *et al.,* 2004 and Jensen *et al*., 1995)

The appropriate system was selected based on optimal resolution with appropriate retention time and total run time. The best HPLC condition for analysis of histamine was examined by varying ratio of mobile phase. These mobile phases were prepared by mixing phosphate buffer (obtained %v/v TEA) : acetonitrile : methanol in the proportions as shown in Table 3-4. Chromatograms of each condition were compared for the condition in which histamine peak well separated from other interfering peaks within an appropriate analysis time.

Table 3-4 Mobile phase composition

Condition	Phosphate buffer (%)	Acetonitrile (%)	Methanol (%)
A	50 (+ 0.5%TEA)	35	15
B	50 (+ 0.5%TEA)	25	25
C	50 (+ 0.1%TEA)	25	25
D	60 (+ 0.1%TEA)	25	15
E	65 (+ 0.1%TEA)	20	15

3.2.8.3 Optimization of derivatization procedure

According to previously reported method for histamine derivatization (Gordon *et al*., 2004, Jensen *et al*., 1995). Histamine solution was mixed with 0.4 M Borate buffer pH 9.5. Derivatizing agent (OPA/ME solution) was subsequently added and the reaction was allowed for min at retention time. The reaction was stopped by phosphoric acid (3.57 N).

Optimization of this procedure was examined according to following variables:

- concentration of derivatizing agent
- volume of OPA
- time before reaction
- reaction time, volume of H_3PO_4
- stability of the compound obtained from derivatization

3.2.8.3.1 Concentrations of derivatizing agent

OPA was dissolved with methanol to yield solutions with concentration of 1%, 0.5%, 0.1% 0.05%, and 0.01% w/v. All solutions were mixed with 2.5 ml/l of 2-ME in a 1:1 (v/v) ratio for optimal result.

3.2.8.3.2 Volume of OPA

Volume of OPA, which mixed with 2.5 ml/l ME, was optimized by varying as 10, 20, 30 and 50 µl for maximum result of histamine derivative.

3.2.8.3.3 Time before reaction

After mixing borate buffer and histamine solution, varying lag time before adding derivatizing agent was done as 0, 2, and 5 min for stability study of mixture.

3.2.8.3.4 Reaction time

Reaction time between histamine and derivatizing agent was varied as 1, 2, 3, 4, 5, and 10 minutes for maximum derivative yield.

3.2.8.3.5 Volume of H_3PO_4

Volume of H_3PO_4 added to stop reaction between histamine and derivatizing agent, were varied to 3.5, 10, 25, and 50 µl.

3.2.8.3.6 Stability of the compound obtained from derivatization

Stability of the compound obtained from derivatization was determined by varying time interval a prior to HPLC injection. The time was test at 0, 5, 10, 15, and 20 minutes accordingly.

3.2.9 Method validation (U.S. Department of Health and Human Services, 2001)

3.2.9.1 Specificity/Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Identification of the peak of histamine was carried out using the test solutions listed in Table 3-5. All solutions were derivatized before analysis and the experiments were determined in triplicate. The analyte should not be interfered from other constituents and well resolved from those constituents.

Table 3-5 A design of specificity testing

Tested item	**MT-HEPES-BSA buffer solution, pH 7.4**	**Compound 48/80**	**Histamine standard solution**	**Rat Peritoneal Mast Cells**	**Ellagic acid rich extract from pomegranate peels**
Blank 1	+	-	-	-	-
Blank 2	-	+	-	-	-
Blank 3	-	-	-	-	+
Control 1	+	-	-	+	-
Control 2	+	+	-	+	-
Control 3	+	+	-	-	-
Standard	+	-	100 ng/ml	-	-
Sample histamine	+	+	-	+	+

+ refers to added in the solution before derivatization

- refers to have not added in the solution before derivatization

3.2.9.2 Limit of detection (LOD) and limit of quantitation (LOQ)

The detection limit of an analytical method is the lowest amount of the analyte that can be detected. LOD value indicates sensitivity of an analytical method. The quantitation limit of an analytical method is the lowest amount of the analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were determined by analysis of serial dilution of the standard histamine solutions, which obtained minimum signal and minimum signal (that can use as lowest for calibration curve), respectively.

3.2.9.3 Linearity/Calibration curve

Calibration curves were constructed by analysis working standard (after derivatization) at different concentrations covering the expected range. The analysis was performed repeatedly for at least 5 determinations per concentration. Peak area was plotted subsequently against the concentration of each reference working standard solutions. The linearity was assessed by using linear regression. The curves were considered linear if coefficient of correlation (R^2) $\geq$ 0.99.

3.2.9.4 Accuracy

The accuracy of an analytical method is the similarity of the test results to the actual value, (i.e. concentration of the analyte). The accuracy of this current analytical method was demonstrated by recovery studies. Three concentrations (low, medium, and high) of working standard solution were prepared as QC samples. These QC samples were analyzed according to the procedure described for calibration curve analysis. Each QC samples concentration was determined in 5 replicates. Recovery was determined using the equation.

$$\text{Recovery} = \frac{C_{obs}}{C_{actual}} \times 100$$

which C_{obs} refers to determined concentration of histamine

C_{actual} refers to actual concentration of standard solution of histamine

3.2.9.5 Precision

Precision of analytical method is the similarity of individual measures of an analyte when the procedure is performed repeatedly. Precision was evaluated based on relative standard deviation (RSD%) determined using the following equation.

$$\text{RSD (\%)} = \frac{\text{SD}}{\text{mean}} \times 100$$

3.2.9.5.1 Within-run analysis

Within-run analysis studies were determined by analysis of QC sample (addressed previously in 3.2.9.4). Each QC sample was determined five times repeatedly. Repeatability of the analytical method was evaluated using RSD (%), which should be lower than 15%.

3.2.9.5.2 Between-run analysis

Between-run analysis studies were determined by analysis of QC sample as same as within-run analysis. Each QC samples was determined five times repeatedly for three time of analysis. Reproducability of analytical method was evaluated using RSD (%), which is considered if %RSD is lower than 15%.

3.2.10 Inhibition of histamine release by ellagic acid (extracted from *Punica granatum* peels)

Inhibitory effect of ellagic acid rich extracts from *Punica granatum* peels, on compound 48/80-induced histamine release was studied. The determination of histamine still released from mast cells was evaluated. Buffer was used as a negative control, and ketotifen was used as a positive control (Table 3-6).

Table 3-6 A design of inhibitory effect study on histamine released by ellagic acid rich extracts

Sample	**MT-HEPES-BSA buffer solution, pH 7.4 (50 µl)**	**Compound 48/80 10 µg/ml (50 µl)**	**DMSO (50 µl)**	**Mast cells (200 µl)**	**Ellagic acid rich extracts (EA) from pomegranate peels (50 µl)**	**Ketotifen (50 µl)**
Control 1 (n=3)	+	-	+	+	-	-
Control 2 (n=3)	+	+	+	+	-	-
Control 3 (n=3)	+	+	-	-	+	+
Sample 1 (n=3)	-	+	-	+	1 µg/ml	0.1 µg/ml
Sample 2 (n=3)	-	+	-	+	10 µg/ml	1 µg/ml
Sample 3 (n=3)	-	+	-	+	100 µg/ml	10 µg/ml
Sample 4 (n=3)	-	+	-	+	1000 µg/ml	-

+ added in the solution

- not added in the solution

3.2.10.1 Preparation of control group

3.2.10.1.1 Control 1 (histamine released from mast cells without stimulation)

The 200 µl of mast cells suspension (about 2.8×10^6 cells/ml) were pipetted into an eppendorf tube and pre-incubated at 37°C for 10 minutes. Fifty microliters of DMSO (as solvent for ellagic acid rich extracts and ketotifen) was added and then incubated at 37°C for 20 minutes. Fifty microliters of MT-HEPES-BSA buffer, pH 7.4 was added and incubated at 37°C for 15 minutes. Incubation tube was then put into a very cool place to stop the reaction.

The cells were separated from the released histamine by centrifugation at 800 rpm for 10 minutes at 4°C and the supernatant was collected. The histamine content was then measured by HPLC analysis.

3.2.10.1.2 Control 2 (histamine released from mast cells stimulated by compound 48/80)

The 200 µl of mast cells suspension (2.8×10^6 cells/ml) were pipetted into an eppendorf tube and preincubated at 37°C for 10 minutes. Fifty microliters of DMSO (as solvent for ellagic acid rich extracts and ketotifen) was added and incubated at 37°C for 20 minutes. 50 µl of 10 µg/ml compound 48/80 was added and incubated at 37°C for 15 minutes. Incubation tube was put into a very cool place to stop the reaction.

The cells were separated from the released histamine by centrifugation at 800 rpm for 10 minutes at 4°C and the supernatant was collected. The histamine content was then measured by HPLC analysis.

3.2.10.1.3 Control 3 (mixed solution of compound 48/80 and ellagic acid rich extracts)

Control 3 was the mixture of compound 48/80 and ellagic acid rich extracts in 1:1(v/v) ratio. Mixed solution was derivatized before injected into HPLC system.

3.2.10.2 Preparation of sample solution

The 200 µl purified mast cell suspensions (2.8×10^6 cells/ml) were pipette and preincubated at 37°C for 10 minutes. Then mast cells were exposed to 50 µl of various concentrations of ellagic acid rich extracts (1 to 1000 µg/ml) or ketotifen (0.1 to 10 µg/ml) for 20 minutes at 3°C immediately prior to stimulation of the cells with 50 µl of compound 48/80 (10 µg/ml) and incubated for an additional 15 minutes. The reaction was stopped by cooling the tubes in ice.

The cells were separated from the released histamine by centrifugation at 800 rpm for 10 minutes at 4°C and the supernatant collected. The histamine content was then measured by HPLC analysis.

The percentage inhibition of histamine release was evaluated using the following equation:

$$\%\text{Inhibition} = \frac{\text{Histamine release without plant extract} - \text{Histamine release with plant extract} \times 100}{\text{Histamine release without plant extract}}$$

3.2.10.3 Assay of histamine release

Histamine released from mast cells was determined by optimized mobile phase and derivatization procedure. First, the supernatant was mixed with 0.4 M H_3BO_4 in ratio 80:20 v/v. Then 30 µl of 0.1% OPA was added into solution and immediately vortex. The reaction time was 4 minutes before adding 50 µl of 3.57 N H_3PO_4 for stopped reaction. The derivatized compound was immediately determined by HPLC analysis which a mixing phosphate

buffer : acetonitrile : methanol in a ratio 60:25:15 v/v/v as mobile phase and flow rate was 1.0 ml/min. The histamine content was detected by fluorescence detection at excitation and emission wavelength as 348 and 444 nm, respectively.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Development of assay for quantitative analysis of histamine

4.1.1 Optimization of mobile phase for HPLC analysis

In the optimized analytical method, analytical method of Gordon *et al*. (2004) and Jensen *et al*. (1995) were modified and optimized by varying ratio of mixture of phosphate buffer (obtained %v/v TEA) : acetonitrile : methanol. ODS C18 column was selected as appropriate stationary phase. All results of HPLC condition are shown in Table 4-1.

Table 4-1 Reults of retention time and total run time of five conditions of HPLC system for optimal separation

Condition	Phosphate buffer (%)	Acetonitrile (%)	Methanol (%)	Total run time (min)	Retention time (min)
A	50 (+ 0.5%TEA)	35	15	15	3.5
B	50 (+ 0.5%TEA)	25	25	25	5.5
C	50 (+ 0.1%TEA)	25	25	25	4.8
D	60 (+ 0.1%TEA)	25	15	20	7.2
E	65 (+ 0.1%TEA)	20	15	30	13.3

Since, histamine was determined at a low level, blank interference was considered as a major problem. All chromatograms are shown in Figure 4-1, which compared between chromatograms of blank and chromatograms of standard histamine solution (1 μg/ml). Histamine standard solution was clearly separated from the others and no interfering peaks under condition D and E of HPLC system. But condition D is the best condition because its run time is the most suitable for routine analytical method. So, the best result is a ratio of phosphate buffer

(obtained 0.1%v/v TEA) : acetonitrile : methanol as 60:25:15 %v/v/v, was obtained base on optimal resolution with appropriate retention time and total run time.

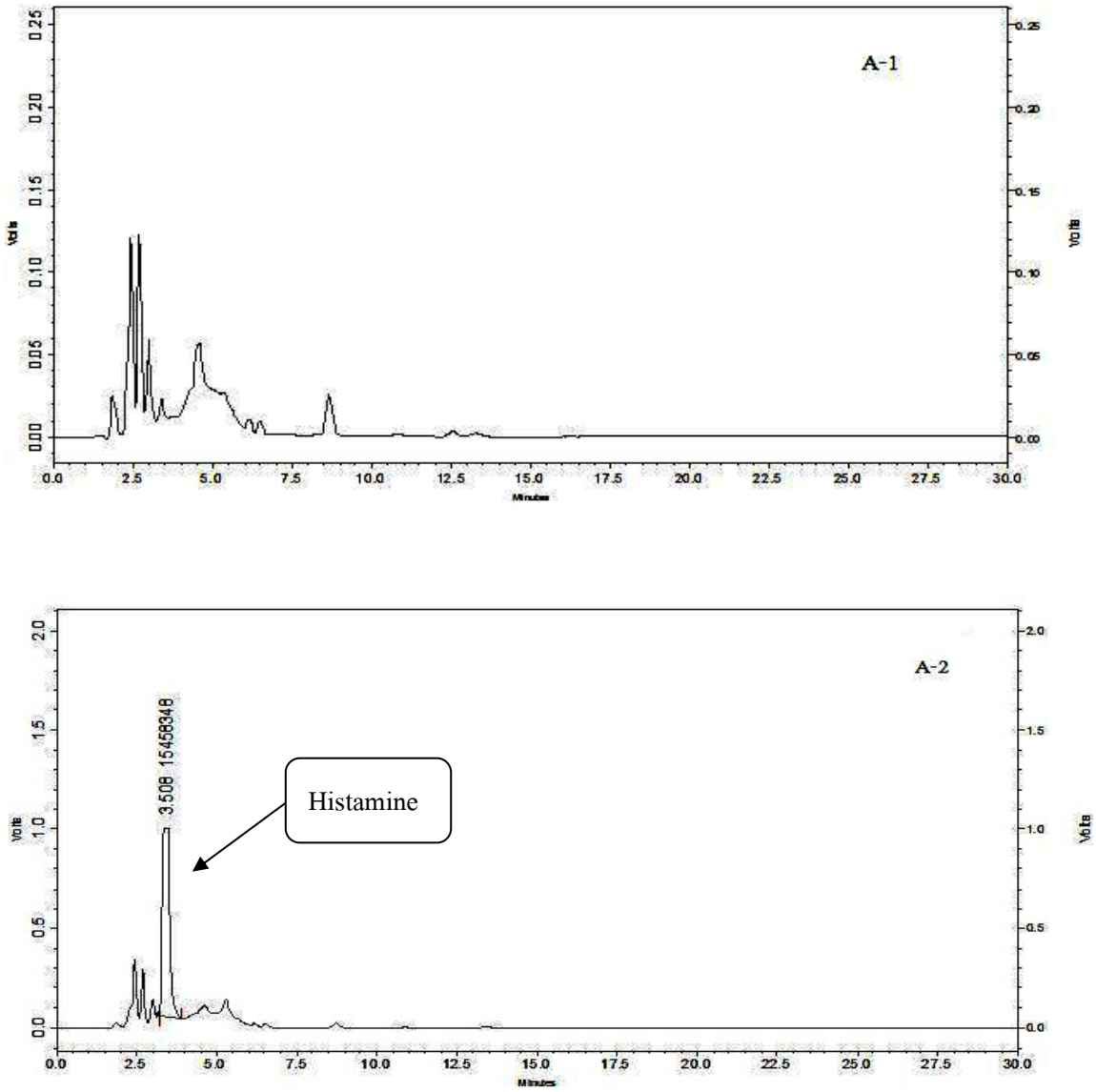


Figure 4-1 Chromatograms of selected conditions of optimal separation for HPLC system; A-1, B-1, C-1, D-1 and E-1 are blank chromatograms of each condition. A-2, B-2, C-2, D-2 and E-2 are chromatograms of standard histamine solution of each condition (1 µg/ml)

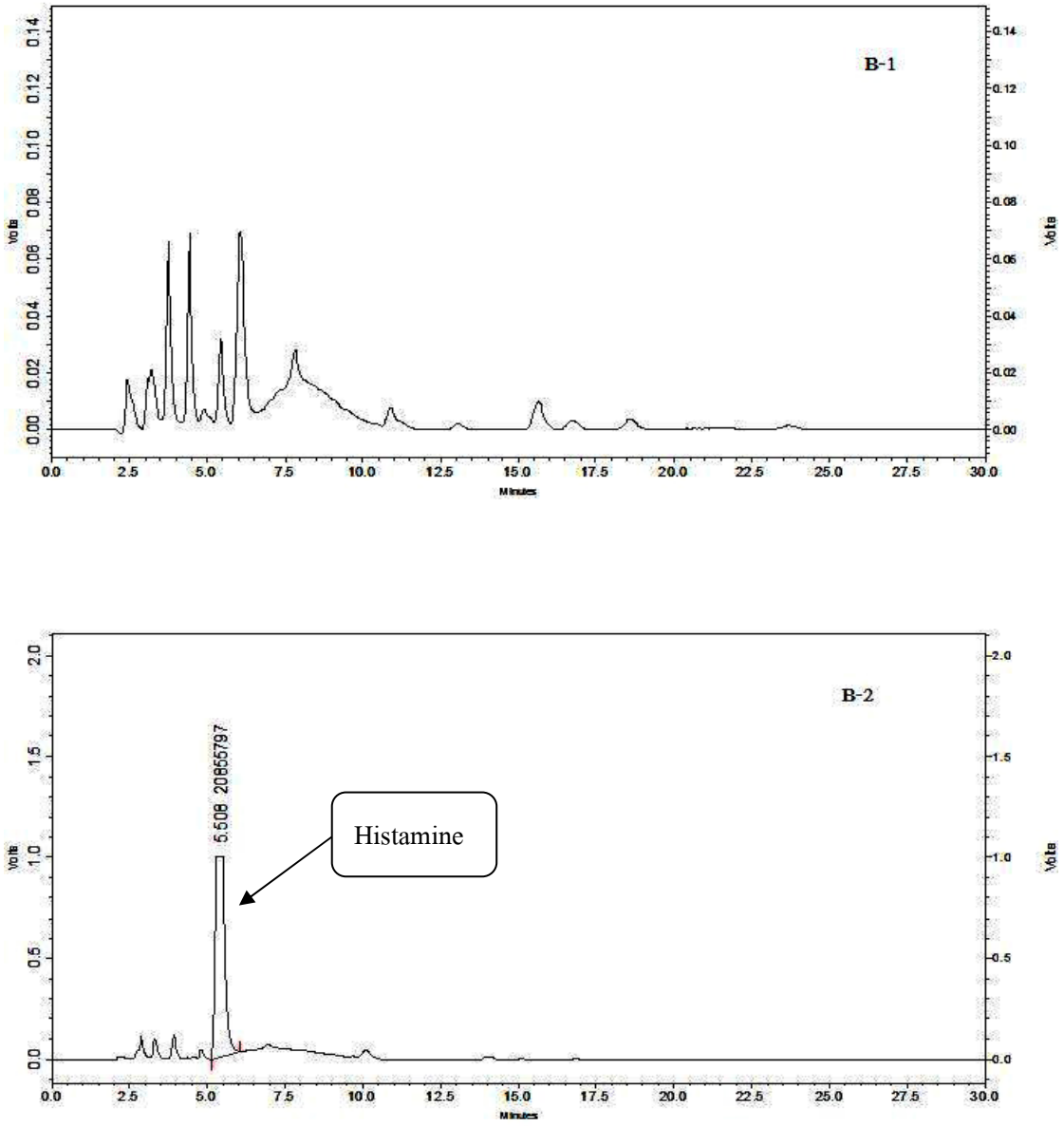


Figure 4-1 (continued) Chromatograms of selected conditions of optimal separation for HPLC system; A-1, B-1, C-1, D-1 and E-1 are blank chromatograms of each condition. A-2, B-2, C-2, D-2 and E-2 are chromatograms of standard histamine solution of each condition (1 µg/ml)

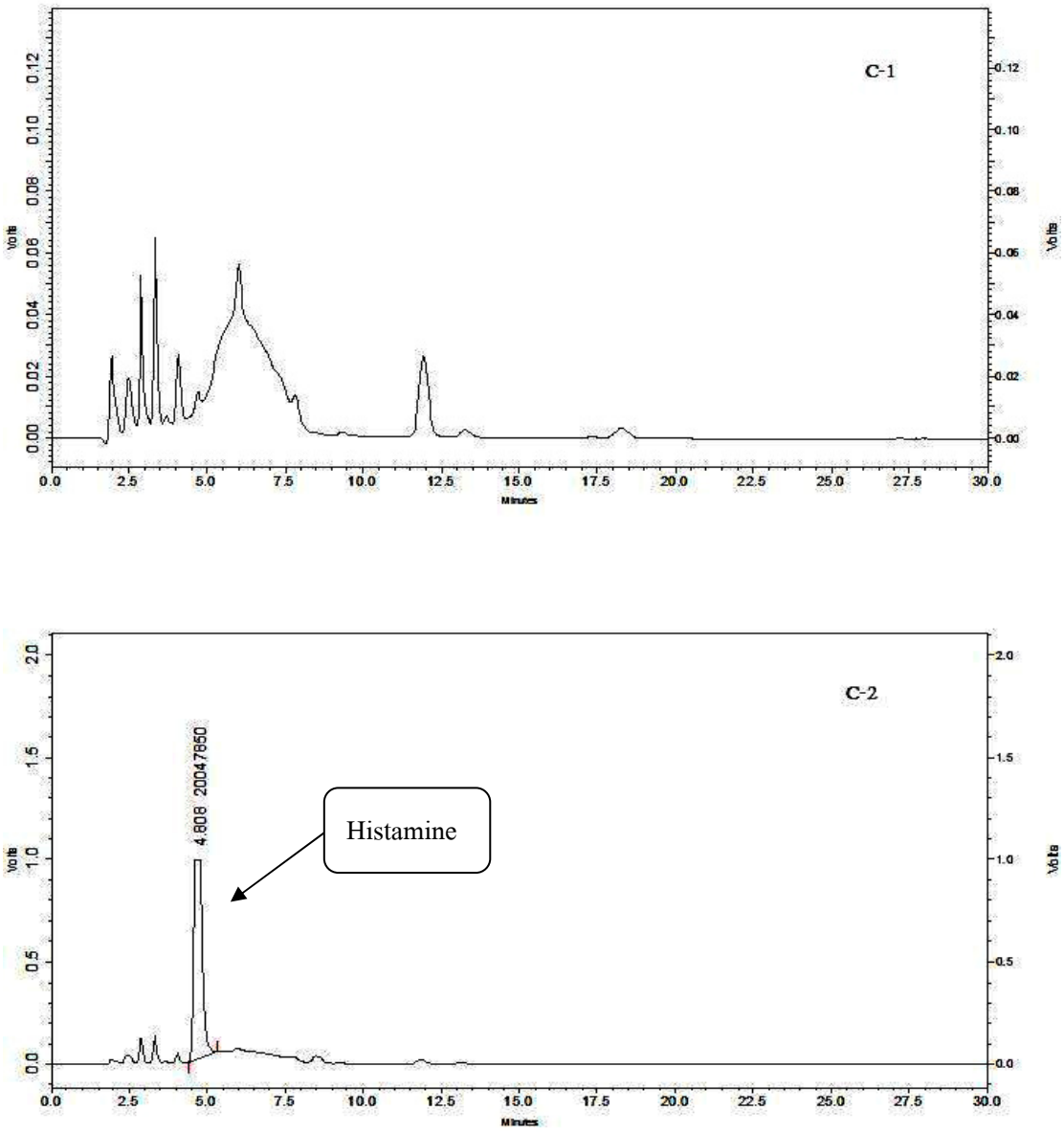


Figure 4-1 (continued) Chromatograms of selected conditions of optimal separation for HPLC system; A-1, B-1, C-1, D-1 and E-1 are blank chromatograms of each condition. A-2, B-2, C-2, D-2 and E-2 are chromatograms of standard histamine solution of each condition (1 µg/ml)

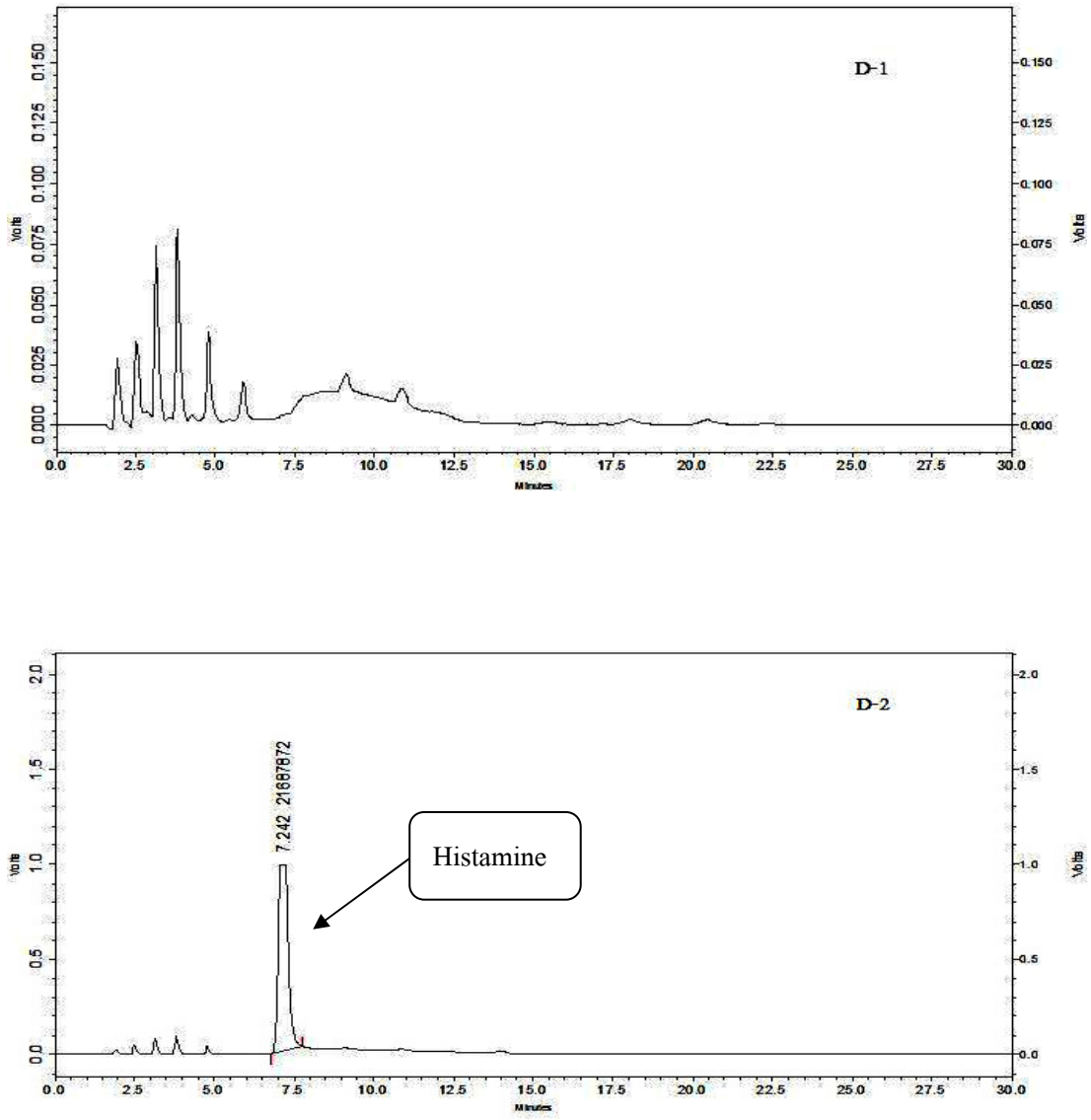


Figure 4-1 (continued) Chromatograms of selected conditions of optimal separation for HPLC system; A-1, B-1, C-1, D-1 and E-1 are blank chromatograms of each condition. A-2, B-2, C-2, D-2 and E-2 are chromatograms of standard histamine solution of each condition (1 µg/ml)

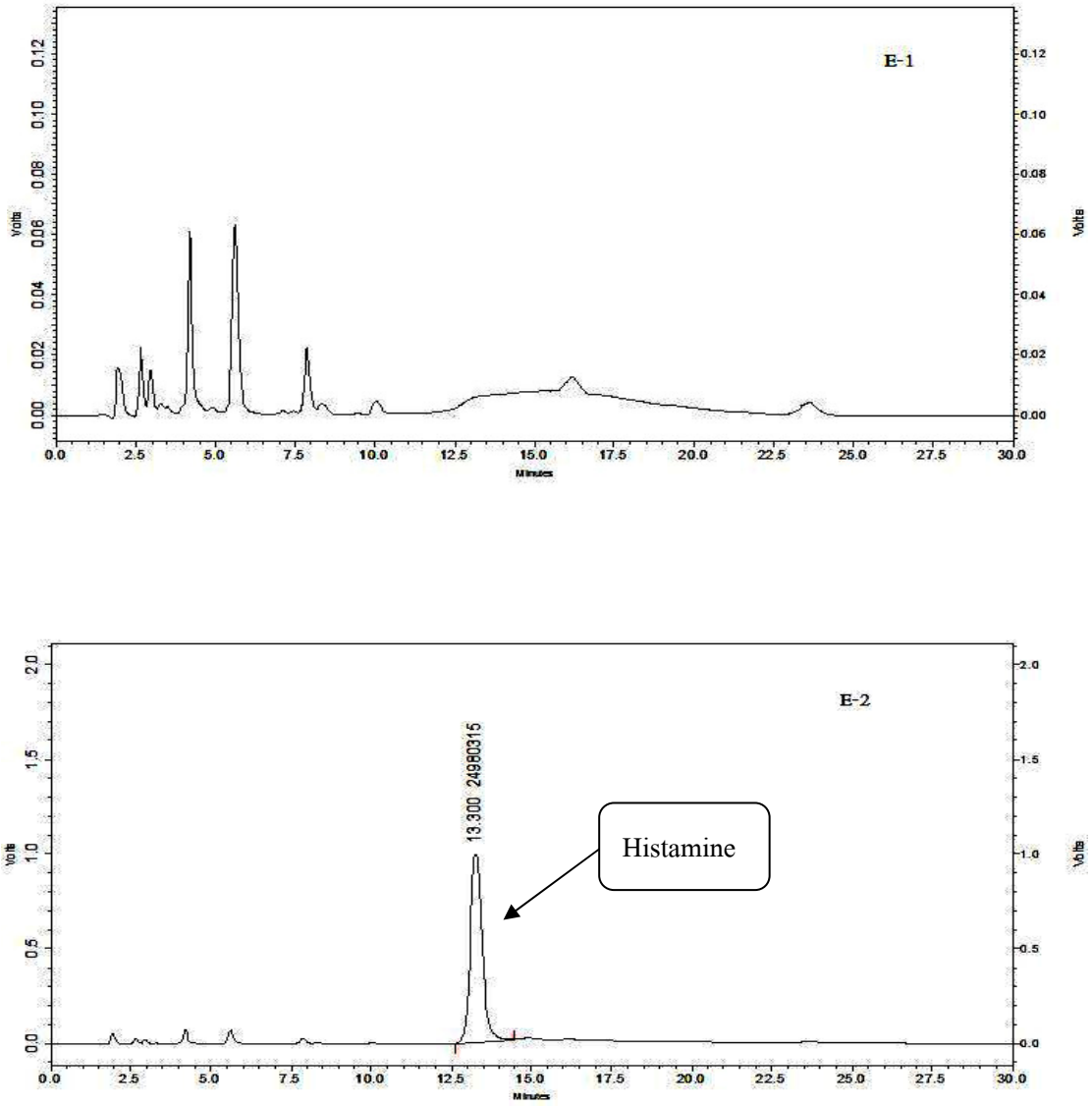


Figure 4-1 (continued) Chromatograms of selected conditions of optimal separation for HPLC system; A-1, B-1, C-1, D-1 and E-1 are blank chromatograms of each condition. A-2, B-2, C-2, D-2 and E-2 are chromatograms of standard histamine solution of each condition (1 μg/ml)

4.1.2 Optimization of derivatization conditions

Since, histamine was determined at a very low level in biological fluid, a derivatization step was optimized to ensure adequate sensitivity before analysis. The best derivatization procedure for analysis of histamine was considered based on the highest peak area of standard histamine determination (50 ng/ml and 100 ng/ml) by varying concentration of derivatizing agent, reaction time, and volume of 3.57 N H_3PO_4 (stopped reaction agent) and test for stability of derivatized compound.

4.1.2.1 Concentration of derivatizing agent

O-phthalaldehyde (OPA) was chosen as a derivatizing agent for histamine determination because OPA was rapidly reacted with primary amine. The results are shown in Table 4-2. OPA concentration was varied at five concentrations for every standard histamine concentration. The sufficient concentration of OPA for derivatized histamine was 0.1%w/v, which achieve the maximum yield at all concentration of standard histamine.

Table 4-2 Effect of OPA concentration on the peak area of histamine.

Concentration of standard histamine	**Peak area of result from varying concentration of derivatizing agent**				
	0.01%	0.05%	0.1%	0.5%	1%
50 ng/ml	762,284	752,038	900,479	540,538	374,642
100 ng/ml	1,453,815	1,452,204	2,109,238	1,008,847	371,394

4.1.2.2 Volume of 0.1%OPA

The volume of 0.1%OPA was optimized by varying a volume as 10, 20, 30 and 50 µl. The results are shown in Table 4-3. It was clear that 30 µl is the most suitable

volume of 0.1%OPA for derivatization of histamine, which achieve the maximum yield at all concentration of standard histamine.

Table 4-3 Effect of volume of 0.1%OPA on the peak area of histamine

Concentration of standard histamine	**Peak area of result from varying volume of 0.1%OPA**			
	10 µl	**20 µl**	**30 µl**	**50 µl**
50 ng/ml	776,604	681,004	1,078,102	364,927
100 ng/ml	1,522,457	1,531,916	1,829,629	971,455

4.1.2.3 Time before reaction

After mixing between standard histamine solution and borate buffer, lag time before reaction was optimized for stability study of a mixture. The results are shown in Table 4-4. The signals were optimum when immediately addition of OPA for reaction. A mixture of histamine solution and borate buffer have not stability, but because borate buffer necessary use in reaction of OPA/ME with histamine. So, addition derivatizing agent was immediately done.

Table 4-4 Effect of lag time before addition OPA for reaction on peak area of histamine.

Concentration of standard histamine	**Peak area of result from varying lag time before addition OPA for reaction**		
	0 min	**2 min**	**5 min**
50 ng/ml	1,078,102	988,887	779,935
100 ng/ml	1,829,629	1,369,170	1,177,615

4.1.2.4 Reaction time

Reaction times (1, 2, 3 , 4, 5 and 10 min) were optimized for derivatization of histamine. The results are shown in Table 4-5. Four minutes is the best reaction time for reaction between histamine and OPA, which achieve the maximum yield at all concentration of standard histamine.

Table 4-5 Effect of reaction time on peak area of histamine.

Concentration of histamine	**Peak area of result from varying reaction time**					
	1 min	**2 min**	**3 min**	**4 min**	**5 min**	**10 min**
50 ng/ml	569,023	466,555	240,759	1,078,102	748,895	440,377
100 ng/ml	1,305,799	1,502,754	491,094	1,829,629	1,441,831	507,465

4.1.2.5 Volume of 3.57 N H_3PO_4

The volume of 3.57 N H_3PO_4 were optimized by varying volume as 3.5, 10, 25, 50 µl. The results are shown in Table 4-6. Fifty microlitres is the best volume of 3.57 N H_3PO_4 for stop reaction between histamine and OPA.

Table 4-6 Effect of volume of 3.57 N H_3PO_4 on peak area of histamine.

Concentration of histamine	**Peak area of result from varying volume of 3.57 N H_3PO_4**			
	3.5 µl	**10 µl**	**25 µl**	**50 µl**
50 ng/ml	545,231	398,403	579,632	1,078,102
100 ng/ml	1,298,234	1,126,180	1,192,374	1,829,629

4.1.2.6 Stability of the compound obtained from derivatization

The stability of compound obtained from derivatization was studied by varying lag time before HPLC analysis. The results are shown in Table 4-7. Immediately analysis after stopped reaction was selected as optimum for derivatization that mean derivatization complex has not stability.

Table 4-7 Effect of lag time before HPLC analysis on peak area of histamine.

Concentration of histamine	**Peak area of result from varying lag time before HPLC analysis**				
	0 min	**5 min**	**10 min**	**15 min**	**20 min**
50 ng/ml	1,895,824	1,877,702	1,690,948	1,231,833	1,174,605
100 ng/ml	3,749,579	2,943,378	1,915,786	2,252,879	1,771,905

4.2 Method validation

4.2.1 Specificity

Method validation was started by selectivity study. Blanks, controls, working of standard histamine solution and sample histamine were evaluated for selectivity. The HPLC chromatograms obtained from the analysis after derivatization are shown in Figure 4-2. They showed that the peak of standard histamine solution and sample histamine solution had appeared at the same retention time, 7.2 min and absence the other peaks in blank and control 3 chromatograms that shown in Table 4-1. It indicating that this method gave satisfactory specificity for determination of histamine released from rat peritoneal mast cells.

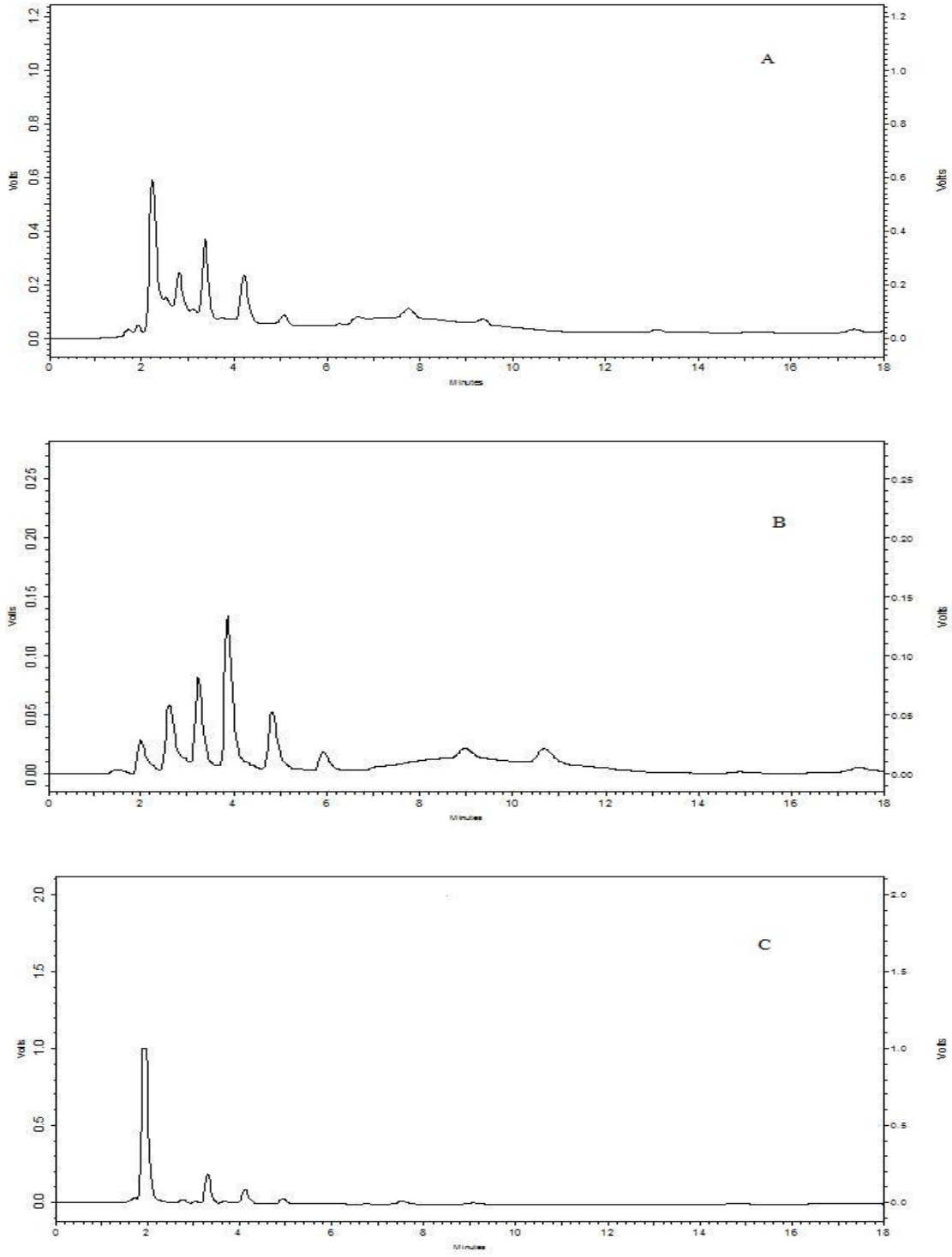


Figure 4-2 HPLC chromatogram of the specificity validation for the HPLC analysis of histamine after derivatization: (A) blank1; (B) blank2; (C) blank3; (D) Control 1; (E) Control 2; (F) Control 3; (G) standard histamine solution 100 ng/ml

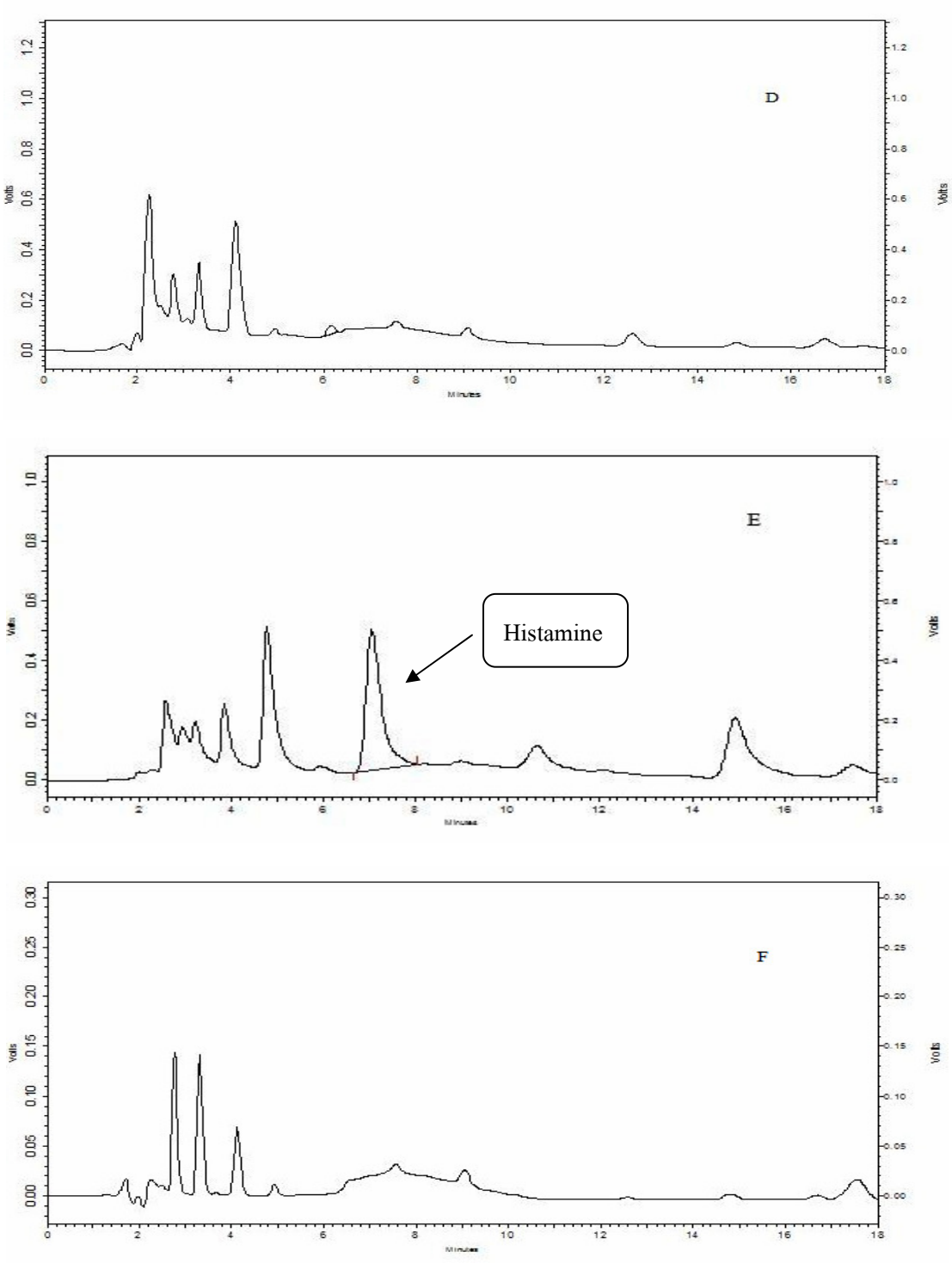


Figure 4-2 (continued) HPLC chromatogram of the validation for the HPLC analysis of histamine after derivatization: (A) blank1; (B) blank2; (C) blank3; (D) Control 1; (E) Control 2; (F) Control 3; (G) standard histamine solution 100 ng/ml

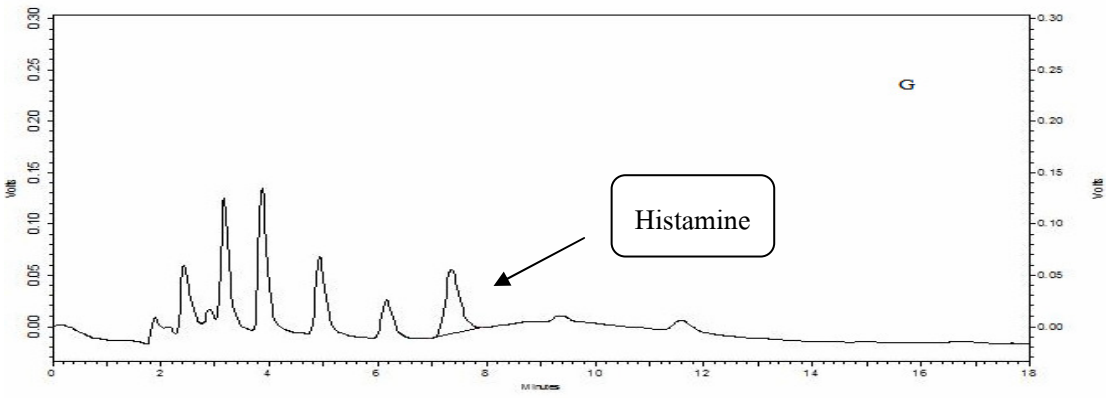


Figure 4-2 (continued) HPLC chromatogram of the specificity validation for the HPLC analysis of histamine after derivatization: (A) blank1; (B) blank2; (C) blank3; (D) Control 1; (E) Control 2; (F) Control 3; (G) standard histamine solution 100 ng/ml

4.2.2 Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection (LOD) and limit of quantitation (LOQ) was estimated by evaluated minimum signal and minimum signal (that can use as lowest for calibration curve), respectively. The chromatograms of LOD and LOQ were presented in Figure 4-3. LOD and LOQ were found to be 5 ng/ml and 10 ng/ml, respectively.

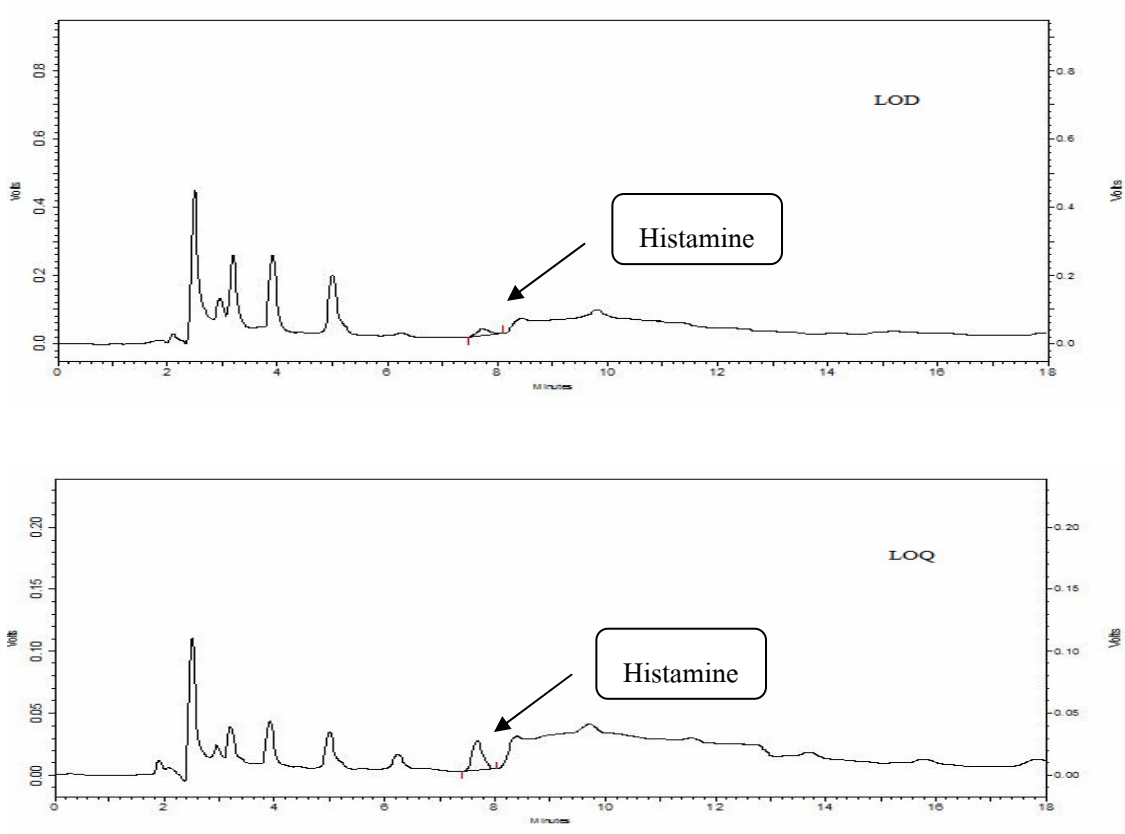


Figure 4-3 HPLC chromatogram of LOD (A) and LOQ (B) analysis

4.2.3 Linearity/Calibration curve and range

Calibration curve for standard histamine solution prepared in mast cell incubation buffer were found to be linear in the concentration range of 10 to 300 ng/ml, when peak area of derivatized histamine standard solutions were plotted against the concentrations of standard histamine solutions. Linearity determination was performed by using five determinations per concentration (10, 25, 50, 100, 200, and 300 ng/ml). The peak area results of calibration curve are shown in Table 4-8. The representative linear equation was y = 47732x – 19441, when y is peak area and x is concentration of standard histamine solution. The correlation coefficient of determination (R^2) is 0.999, is considered acceptable for biological analysis. Regression plot and correlation coefficient are shown in Figure 4-4.

Table 4-8 Peak area for calibration curve

Concentration of standard histamine solution (ng/ml)	Peak area of derivatized histamine					average	SD	RSD (%)
	N1	N2	N3	N4	N5			
10	310,723	386,679	347,322	335,189	366,472	349,277	29,083	8.33
25	964,243	990,981	989,553	935,060	944,455	964,858.4	25,479.6	2.64
50	2,008,061	2,154,758	2,185,266	2,312,056	2,314,964	2,195,021	127,234	5.80
100	4,478,510	4,958,486	4,443,144	4,268,293	4,753,307	4,580,348	273,623	5.97
200	8,943,070	9,452,718	8,772,622	9,348,329	9,696,592	9,242,666	378,165	4.09
300	14,622,362	14,270,644	14,237,801	13,606,726	14,250,260	14,197,559	367,178	2.59

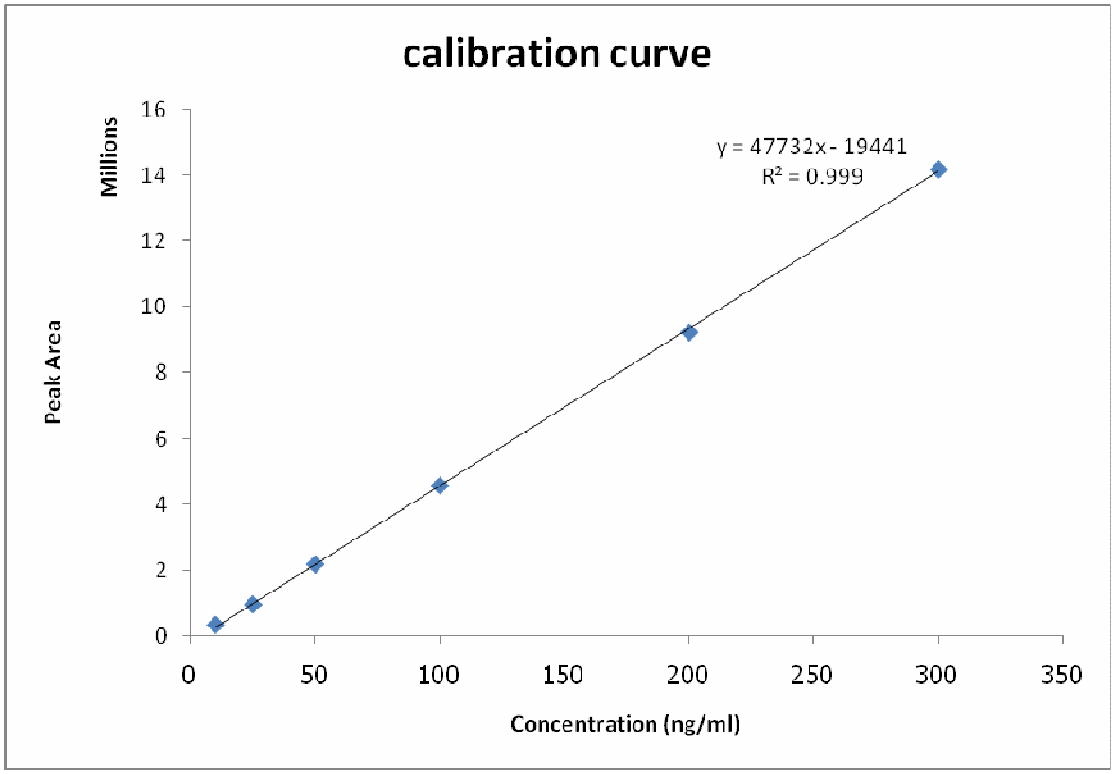


Figure 4-4 Calibration curve for standard histamine solution analysis

4.2.4 Accuracy

The accuracy of an analytical method was assessed by recovery studied. The %recovery was between 97.28 and 99.15 as shown in Table 4-9. It showed that the proposed analytical method has good accuracy with %RSD < 15%.

Table 4-9 Results of accuracy determination by recovery studied.

Spiked level (ng/ml)	**Recovery (%)[a]**			**Mean (%)**	**RSD (%)[b]**
	Day 1	**Day 2**	**Day 3**		
25.15	99.60±0.28	97.53±0.75	94.71±0.32	97.28	2.52
100.61	94.44±8.69	107.74±1.41	95.27±4.16	99.15	7.51
201.22	99.93±1.42	97.67±9.49	96.23±4.47	97.94	1.91

[a]mean±SD; [b]RSD (%) = (SD/mean) × 100

4.2.5 Precision

4.2.5.1 Within-run precision

The within-run analysis was assessed by analysis at three concentrations: 25.15, 100.61, and 201.22 ng/ml of standard histamine solution (n=3). The RSD (%) were 1.13, 9.15, and 0.71%, respectively as shown in Table 4-10. It shows good within-run precision revealed %RSD < 15.

4.2.5.2 Between-run precision

The between-run analysis was assessed for three days by analysis at three concentrations: 25.15, 100.61, and 201.22 ng/ml of standard histamine solution (n=9). The RSD (%) were 2.52, 7.51, and 1.90%, respectively as shown in Table 4-10. It shows good within-run precision revealed %RSD < 15.

Table 4-10 Summary of within- and between-run precision data for determination of standard histamine

Concentration (ng/ml)	Within-run (n=3)		Between-run (n=9)	
	Mean[a]	RSD[b] (%)	Mean[a]	RSD[b] (%)
25.15	25.05±0.28	1.13	24.47±0.62	2.52
100.61	95.02±8.69	9.15	99.76±7.49	7.51
201.22	201.08±1.42	0.71	197.08±3.76	1.90

[a]mean±SD; [b]RSD (%) = (SD/mean) × 100

4.3 Histamine release inhibition study by ellagic acid (extracted from *Punica granatum* peels)

The pathological mechanism involved Type-I allergy has been explained as the degranulation of mast cells, followed by the release of mediators such as histamine, leukotrienes and prostaglandins. Histamine is the most important mediator for allergy. The degranulation of mast cells occurs in response to the immunological stimuli in which the antigen-antibody reaction on the mast cell surface. Therefore, inhibition for degranulation of mast cell was studied. Ellagic acid (EA) rich extracts from *Punica granatum* peels were studied for inhibitory effect on compound 48/80 induced histamine released from mast cells. Using ketotifen as a positive controls, results were summaried in Table 4-11 and Figure 4-5.

Table 4-11 %Inhibition of ellagic acid rich extracts on mast cell degranulation

Sample (µg/ml)	Peak area of derivatized histamine				average	Substract from control 1	% Inhibition
	N1	N2	N3	N4			
EA 1	14,703,460	14,903,546	15,489,839	15,549,433	15,161,569	6,101,816	7.66
EA 10	11,692,829	11,693,773	14,905,796	15,279,621	13,393,004	4,333,251	34.42
EA 100	13,531,229	13,743,051	11,194,304	11,115,462	12,396,011	3,336,258	49.51
EA 1000	10,283,689	10,117,505	11,572,062	11,838,723	10,952,994	1,893,241	71.35
Control 1	8,291,372	8,444,952	10,011,332	9,491,358	9,059,753	0	-
Control 2	19,002,993	18,648,038	1,248,240	12,591,613	15,667,721	6,607,967	-

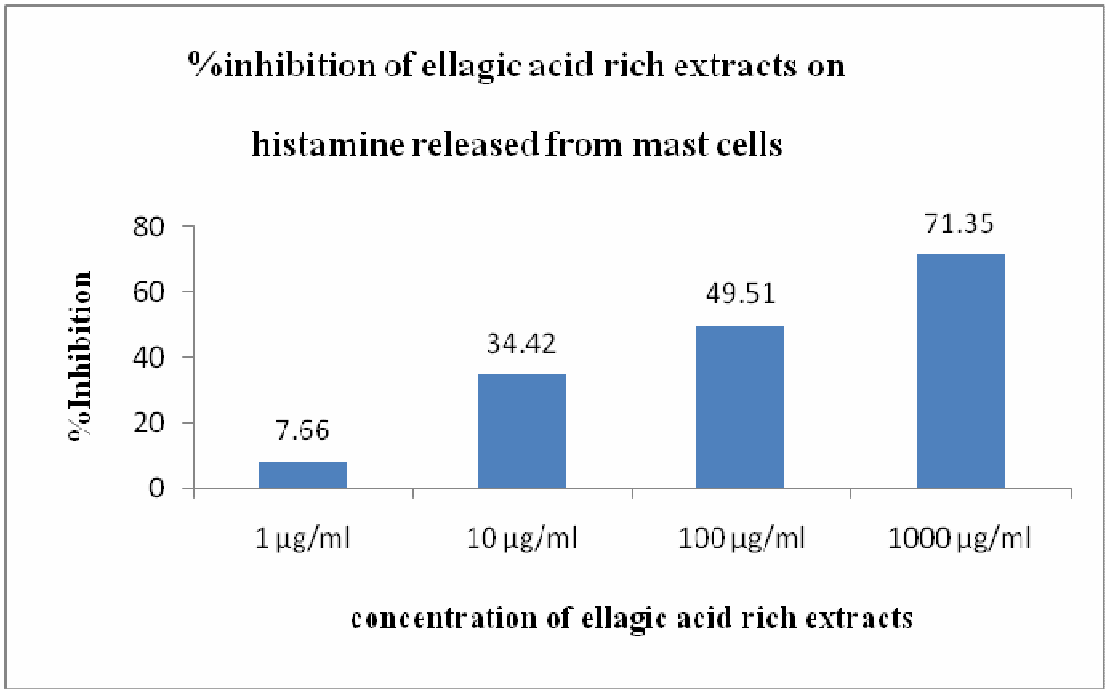


Figure 4-5 Concentration dependence inhibition of ellagic acid on mast cell degranulation

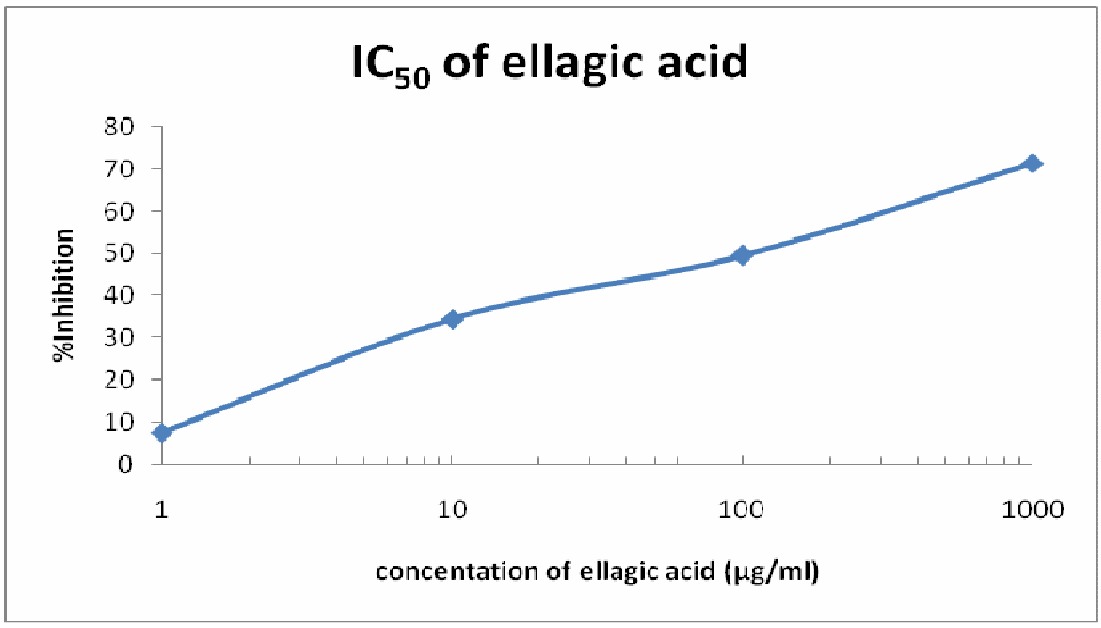


Figure 4-6 IC_{50} determination method of ellagic acid on mast cell degranulation

The present investigation indicates that ellagic acid rich extracts from *Punica granatum* peels is active in Type-I allergic conditions because its ability to inhibited the degranulation of mast cells in concentration range of 1-1,000 µg/ml. Comparison to a positive control, ketotifen ability to inhibited the degranulation of mast cells in concentration range of 0.1-10 µg/ml as shown in Table 4-12 and Figure 4-7. The IC_{50} for ketotifen was 0.56 µg/ml, while 87.09 µg/ml as IC_{50} of ellagic acid that shown in Figure 4-6 and 4-8. So, inhibitory effect study of ellagic acid rich extracts as a model of Thai herbal crude extract for inhibitory effect study on histamine released from mast cells was successfully established by this analytical method.

Table 4-12 %Inhibition of ketotifen on mast cell degranulation

Sample (µg/ml)	Peak area of derivatized histamine				average	Substract from control 1	% Inhibition
	N1	N2	N3	N4			
ketotifen 0.1	5,406,899	5,639,955	6,079,448	5,805,411	5,732,928.2	3,021,315	31.39
ketotifen 1	3,922,916	4,284,075	4,636,633	5,298,038	4,535,415.5	1,823,802.	58.58
ketotifen 10	3,734,427	4,177,268	3,133,671	3,071,363	3,529,182.2	817,569	81.43
Control 1	2,406,069	3,140,324	2,844,681	2,455,379	2,711,613.2	0	-
Control 2	7,610,826	7,446,220	5,997,078	7,406,570	7,115,173.5	4,403,560.2	-

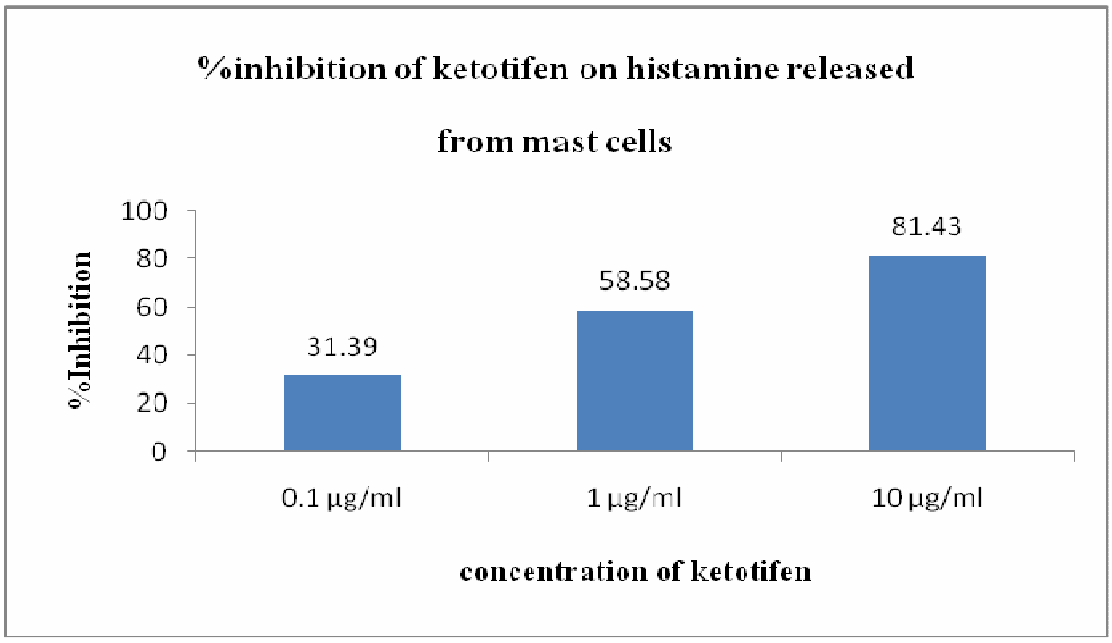


Figure 4-7 Concentration dependence inhibition of ketotifen on mast cell degranulation

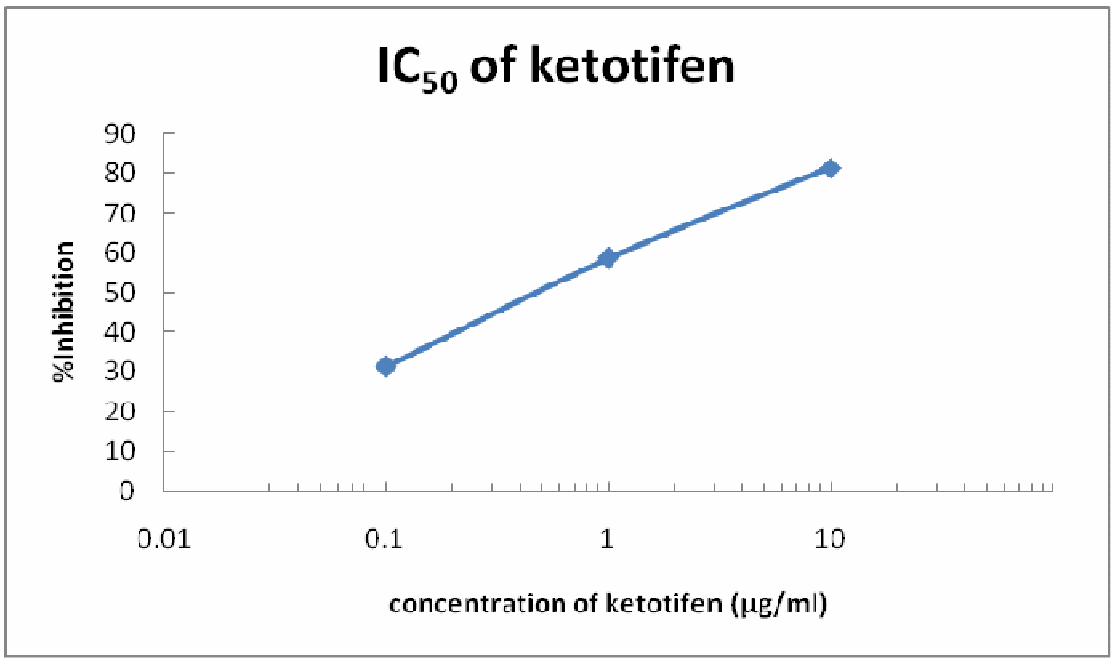


Figure 4-8 IC_{50} determination method of ketotifen on mast cell degranulation

CHAPTER 5

CONCLUSION

Quantitative analysis of histamine was successfully developed and validated. Using reverse-phased high performance liquid chromatographic method with fluorescence detection (348 nm and 444 nm for excitation and emission wavelength, respectively), the method was simple, rapid, accurate and precise. A mixture of phosphate buffer (obtained 0.1%v/v TEA) : acetonitrile : methanol in a ratio 60 : 25 : 15 %v/v/v, pH 6.4 was employed in the isocratic mode with a solvent delivery system at a flow rate of 1.0 ml/min as a mobile phase. Pre-column derivatization of histamine was required before analysis by ODS-C18 column. Derivatization procedure initiated by alkalinizing histamine solution with 0.4 M H_3BO_4. Then, 30 µl of 0.1% OPA was added into solution and the reaction was allowed for 4 minutes at retention time. The reaction was terminated by addition of phosphoric acid. The derivatized compound was instantly injected onto by HPLC column to prevent degranulation of the derivatives.

This method was sensitive with a detection limit of 5 ng/ml. Linearity was fixed in range concentration as 10 to 300 ng/ml. Calibration curve were drawn between the peak area versus concentration of histamine. The slope and correlation coefficient value were y = 47,732x – 19,441 and 0.999, respectively. Accuracy of the method was evaluated by %recovery, which ranged was between 97.28 and 99.15%. Within-run analysis of three concentrations showed acceptable results with %RSD = 1.13, 9.15, and 0.71%. Between-run analysis of three concentration revealed good between-run precision with %RSD = 2.52, 7.51, and 1.90%, respectively. The %RSD of both within-run and between-run were within 15% indicating good precision of the method.

Application of the developed method was demonstrated in the study of the inhibitory effect of ellagic acid on histamine released from mast cells. Ellagic acid inhibited histamine released from rat-peritoneal mast cell in concentration dependent mannar. By in studying histamine release, IC_{50} of ellagic acid was 87.1 µg/ml. Therefore, the developed method was proved to be sufficient and applicable to determine histamine in physiological-relvant level.

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