Journal of Chromatography, 617 (1993) 233–240
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6915

Rapid and highly sensitive high-performance liquid chromatographic method for the determination of histamine and 3-methylhistamine in biological samples using fluorescamine as the derivatizing agent

Charles M. C. J. van Haaster*, Wim Engels and Paul J. M. R. Lemmens

Cardiovascular Research Institute Maastricht, University of Limburg, P.O. Box 616, 6200 MD Maastricht (Netherlands)

Gerard Hornstra

Department of Human Biology, University of Limburg, Maastricht (Netherlands)

Ger J. van der Vusse

Department of Physiology, University of Limburg, Maastricht (Netherlands)

(First received April 2nd, 1993; revised manuscript received May 17th, 1993)

ABSTRACT

A highly sensitive and rapid high-performance liquid chromatographic assay for the determination of histamine and 3-methylhistamine in biological samples using 1-methylhistamine as the internal standard is described. Samples were purified and concentrated on cation-exchange columns and derivatized with fluorescamine. The lower detection limit was 20 pg on-column. Linearity was demonstrated up to 20 ng on-column. The samples could be derivatized simultaneously before injection and were stable for 7 days. The method was used for the determination of histamine and related compounds in coronary perfusates, extracts of homogenized rat hearts, and supernatants of stimulated peritoneal mast cells.

INTRODUCTION

Histamine, an inflammatory substance present in granules of mast cells and peripheral blood basophils, plays an important (patho)physiological role in allergy, inflammation, gastric acid secretion, microcirculation and neurotransmission [1,2]. Histamine is capable of inducing a wide variety of both physiological and pathological alterations in various tissues and cell types. Therefore, a reliable and sensitive technique to assess histamine in biological samples is highly relevant, especially for studies concerning the role of endogenous histamine on cardiac function in ischemia/reperfusion or hypoxia/reoxygenation conditions, where an assay suitable for analysing small amounts of histamine in coronary perfusates is required.

In general, histamine is assessed by fluorometry [3], bioassays [4], gas chromatography [5] and high-performance liquid chromatography (HPLC) [6–14]. All HPLC techniques described have the disadvantage of either the relative in-

* Corresponding author.

0378-4347/93/$06.00

stability of the derivatives, the need for carefully controlled reaction conditions (derivatization with *o*-phthalaldehyde, OPA) or a lack of the addition of an internal standard, or the need for sophisticated HPLC equipment. Because concentrations of histamine in biological samples can be very low, and many biological compounds might interfere in the histamine assay [15,16], a cleanup and a concentration step prior to analysis should be introduced.

We have developed a rapid and simple isocratic HPLC method for the determination of histamine using fluorescamine as the derivatizing agent. The procedure involves a clean-up/concentration step on carboxylic acid cation-exchange columns, followed by derivatization with fluorescamine using 1-methylhistamine as the internal standard. Because the presence of 3-methylhistamine in heart homogenates has been described [17,18], we also included the determination of 3-methylhistamine in our method, which has been used to measure histamine in coronary perfusates, and also to determine histamine and related compounds in rat heart homogenates and supernatants of stimulated rat peritoneal mast cells.

EXPERIMENTAL

Materials

Histamine, 3-methylhistamine, 1-methylhistamine and compound 48/80 (a condensation product of N-methyl-*p*-methoxyphenethylamine with formaldehyde) were obtained from Sigma (St. Louis, MO, USA). Bakerbond carboxylic acid (COOH) 40 μm, 60 Å, bulk package, was obtained from Baker (Philipsburg, NJ, USA). $NaH_2PO_4 \cdot H_2O \cdot HCl$ (Suprapur), Na_2EDTA, NH_4OH, *o*-H_3PO_4 and $HClO_4$ (all analytical grade) were obtained from Merck (Darmstadt, Germany). Acetonitrile and methanol (HPLC grade) were purchased from Rathburn (Walkerburn, UK). Triethylamine (analytical grade) was obtained from Janssen Chimica (Tilburg, Netherlands). Fluorescamine was from Fluka (Buchs, Switzerland). Water was purified with a Milli-Q purification unit from Millipore (Bedford, MA, USA). Triethylamine was purified on short tip-plugged Pasteur pipettes with Al_2O_3, alumina N grade super I (ICN Biomedicals, Costa Mesa, USA).

Purification and concentration of the samples

Small columns were prepared with a slurry of Bakerbond carboxylic acid material in methanol (150 mg/column) in Pasteur pipettes, tip-plugged with glassfibre prefilter material (Sartorius, Göttingen, Germany). The columns were washed with 2 ml of water followed by 2 ml of 1 *M* HCl, 2 ml of water, and 4 ml of 0.2 *M* sodium phosphate buffer (pH 6.4). After addition of 1-methylhistamine as the internal standard, samples (brought to pH 6.4 with phosphate buffer) were applied to the columns. Columns were washed with 4 ml of 50 m*M* Na_2EDTA (pH 6.4) and 4 ml of water. Histamine, 3-methylhistamine and the internal standard were eluted with 1 ml of 1 *M* HCl. The eluate was dried for *ca.* 15 min under a stream of nitrogen at 40°C, and the residue was dissolved in 100 μl of water.

Derivatization

To the purified samples, 400 μl of 50 m*M* $Na_2B_4O_7$ (pH 9.1) were added. Under continuous, vigorous stirring, 500 μl of a freshly prepared fluorescamine solution in acetonitrile (20 mg/100 ml) were added. After stirring for 1 min, the derivatized samples were dried under a stream of nitrogen at 40°C. The residues were dissolved in 1 ml of the HPLC mobile phase (see below).

HPLC instrumentation

The HPLC system consisted of a Spectroflow 400 pump (Kratos Analytical, Ramsey, USA), a Promis auto-injector (Spark Holland, Netherlands) with a Rheodyne 7010 injection valve, and a Perkin-Elmer LS-1 fluorescence detector (Beaconsfield, UK) with a reversed-phase C_{18} column, 250 × 4 mm I.D., 5 μm particles (Chrompack, Middelburg, Netherlands). The injection volume was 20 μl, and the flow-rate was 0.7 ml/min. The fluorescence detector had the following settings: excitation wavelength, 360 nm; emission

wavelength, 440 nm. The temperature of the HPLC column was kept at 20°C. Samples were kept at 4°C in the auto-injector device in the dark. Data were acquired with Rainin software, using the Dynamax program for integration of the chromatograms (Rainin Instrument, Ridgefield, USA).

Chromatographic conditions

The mobile phase was water–acetonitrile–triethylamine–H_3PO_4 (850:150:4:4, v/v) brought to pH 6.4 with NH_4OH. To obtain the optimum response of the fluorescence detector and a low noise level, the mobile phase was degassed daily with helium gas (5 min at a flow-rate of 250 ml/min for each 1000 ml of the mobile phase).

Assessment of linearity, stability, reproducibility and recovery

The relationship between the amounts of fluorescamine-derivatized histamine or 3-methylhistamine injected on column and their peak areas was assessed as follows. To 1 ml of 0.2 *M* phosphate buffer (pH 6.4), various amounts of histamine and 3-methylhistamine standard solutions (0, 100, 200, 300, 400, 500, 600 and 700 ng, each amount prepared in triplicate) were added. To all samples 500 ng of the internal standard 1-methylhistamine was added. After concentration and derivatization of the samples, the derivatives were dissolved in 1 ml of the HPLC mobile phase. Following injection of 20 μl of the samples (corresponding to 0–14 ng on column) and separation by HPLC, the absolute peak areas of histamine and 3-methylhistamine were transformed to relative peak areas by correction for the internal standard. Samples were analysed immediately after derivatization and re-analysed on days 1 and 7.

To determine the stability, samples (n = 12) from a standard solution containing histamine (300 ng), 3-methylhistamine (300 ng) and 1-methylhistamine (250 ng) were concentrated, derivatized and analysed by HPLC on days 0 and 7.

To determine the reproducibility, two standard solutions (I and II) were prepared. Standard solution I contained 1500 ng/ml histamine, 1500 ng/ml 3-methylhistamine and 3000 ng/ml the internal standard. Standard solution II contained 6000 ng/ml histamine, 6000 ng/ml 3-methylhistamine and 3000 ng/ml internal standard. On three consecutive days, 100-μl samples (n = 6) were withdrawn from the solutions I and II. Samples were brought onto the cation-exchange columns in 2 ml of 0.2 *M* phosphate buffer, (pH 6.4). After derivatization, samples were immediately analysed by HPLC.

To determine the recovery of the purification/concentration procedure, 100-μl samples from solution I (n = 10) were immediately derivatized with fluorescamine, solubilized in 1 ml of the mobile phase, and analysed by HPLC. The absolute peak areas of these samples were compared with the absolute peak areas from the samples concentrated on the cation-exchange columns.

Rat heart homogenates

To determine the total amount of histamine and 3-methylhistamine in rat heart homogenates, heparinized (1000 I.U./kg intraperitoneally) male Lewis rats (mean weight 280 ± 25 g, n = 9) were killed by decapitation. After thoracotomy, hearts were rapidly excised and immediately perfused by the Langendorff technique at a constant pressure (80 mmHg). The perfusion fluid was Krebs–Ringer's (KR) solution (pH 7.4) containing (in m*M*) NaCl 130, KCl 5.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 28.6, $CaCl_2$ 2.2, and glucose 11.1. The buffer was kept at 37°C and was equilibrated with O_2–CO_2 (95:5) throughout the experiment. Hearts were perfused for 15 min. After removal of the atria, hearts were weighed and finely minced, and 500 ng of the internal standard were added. Hearts were homogenized in ice-cold 50 m*M* Tris–HCl buffer (pH 8.5) with the use of an Ultra-Turrax (Wilten Woltil, Netherlands). Thereafter, samples were sonicated (30 W, amplitude 12 μm, peak to peak) with an MSE sonifier (Crawley, UK) for 2 min with repeated intervals of 5 s. After addition of $HClO_4$ to a final concentration of 0.3 *M*, samples were boiled for 5 min at 100°C. Samples were neutralized with KOH, cooled to 0°C and centrifuged for 5 min at 2000 *g* at 4°C to remove the precipitate of $KClO_4$. Su-

pernatants were collected and stored at −20°C until analysis.

Samples of coronary perfusates

In a subset of experiments, histamine and 3-methylhistamine were determined in coronary perfusates collected from hearts of male Lewis rats (mean weight 275 ± 20 g, $n = 3$). Degranulation of cardiac mast cells was obtained by a bolus injection of 50 μg of compound 48/80. Coronary effluent was collected during the following period of 1 min.

Samples of peritoneal mast cells

Mast cells were obtained by peritoneal lavage of a male Wistar rat as described previously [19]. Mast cells (final concentration $1 \cdot 10^5$ cells/ml) were stimulated with 10 μg/ml compound 48/80 in the presence of 1 m*M* $CaCl_2$, and incubated for 15 min at 37°C. Blank histamine release was obtained by stimulation with phosphate-buffered saline (PBS). To separate the mast cells from the supernatant, samples were layered onto silicon oil and centrifuged at 10 000 *g* for 1 min. To the supernatant 500 ng of the internal standard were added. Samples were stored at −20°C until analysis. Incubation with 0.1% Triton X-100 (v/v) was used to determine the total content of mast cell histamine.

RESULTS

Linearity, stability, reproducibility and recovery

The linearity of the fluorescence intensity *versus* the amounts of histamine and 3-methylhistamine was evaluated by analysing a range from 0 to 700 ng of histamine and 3-methylhistamine per sample (corresponding to 0–14 ng on column). Plots of relative peak areas of histamine and 3-methylhistamine *versus* the amounts derivatized were linear ($r = 0.996$ and 0.999, respectively). Regression equations of relative peak areas (y) *versus* the amounts (on column) of derivatized histamine and 3-methylhistamine (x) were $y = 0.090x + 0.056$ and $y = 0.116x + 0.031$, respectively. Because the samples were individually concentrated, derivatized and analysed, these results show that the procedure has a constant efficiency, which is independent of the amount of histamine and 3-methylhistamine derivatized over the range selected. In practice, linearity was demonstrated up to 20 ng on column for both histamine and 3-methylhistamine (data not shown). Histamine and 3-methylhistamine could be detected up to 20 pg on column at a signal-to-noise ratio of 3:1. Analysis of a standard solution containing 300 ng of histamine and 3-methylhistamine in the presence of 250 ng of the internal standard (see Experimental) on day 7 demonstrated that only 10% of the fluorescence intensity is lost over a period of 7 days. Absolute peak areas of histamine, 3-methylhistamine and the internal standard on day 7 (as a percentage of the absolute peak areas on day 0) were 90.9 ± 4.5%, 91.7 ± 2.9% and 90.9 ± 2.6%, respectively.

Derivatives of standard solutions used for the calibration curves were analysed immediately following derivatization and re-analysed on days 1 and 7. Multiple analysis of variance revealed no significant differences between the relative peak areas of histamine and 3-methylhistamine on days 0, 1 and 7 ($\alpha = 0.05$) for all the amounts used.

Results from the determination of the reproducibility are presented in Table I. The recovery of the concentration procedure was greater than 95%, because absolute peak areas of concentrated samples were always greater than 95% of those not concentrated on the cation-exchange columns.

Applications

The combination of purification of samples on cation-exchange columns followed by derivatization with fluorescamine was used to determine histamine and 3-methylhistamine in various biological samples. Fig. 1A shows a typical chromatogram of a standard solution containing 5 ng (on column) of histamine, 3-methylhistamine and the internal standard, showing that histamine elutes at 15 min, 3-methylhistamine at 20 min and the internal standard at 25 min. All compounds were completely separated.

Fig. 1B shows a typical chromatogram of a

TABLE I

REPRODUCIBILITY DATA FOR THE DETERMINATION OF HISTAMINE AND 3-METHYLHISTAMINE IN STANDARD SOLUTIONS

Each sample (six for each concentration) was concentrated on the cation-exchange columns, derivatized with fluorescamine and analysed by HPLC on three days in a one-week period. Standard solutions were prepared as described in Experimental. The coefficients of variation (C.V.) were obtained by dividing the standard deviation by the mean.

Concentration (ng/sample)	C.V. (%)			
	Within-day C.V.[a]		Day-to-day C.V.[b]	
	Histamine	3-Methylhistamine	Histamine	3-Methylhistamine
150	3.2	3.3	2.9	2.7
600	6.7	6.9	6.7	5.9

[a] $n = 6$.
[b] $n = 3$.

coronary perfusate collected during a 1-min period after a bolus injection of 50 μg of compound 48/80. The degranulation of cardiac mast cells resulted in a net release of 1550 ± 150 ng (mean ± S.D.) of histamine in this time interval. No 3-methylhistamine could be detected in the coronary perfusate. In coronary perfusates of non-stimulated hearts, both histamine and 3-methylhistamine were undetectable.

Fig. 1C shows a representative chromatogram of a rat heart homogenate. The presence of 3-methylhistamine in rat hearts could not be demonstrated, but the average amount of histamine was 3.7 ± 0.4 μg/g wet weight (mean ± S.D.). When rat heart homogenates ($n = 3$) were spiked with 500 ng of 3-methylhistamine, we observed average amounts of 480 ± 20 ng, corresponding to 96% recovery of this compound.

Stimulation of rat peritoneal mast cells with 10 μg/ml compound 48/80 (Fig. 1D) in the presence of 1 m*M* $CaCl_2$ resulted in the release of 18 ± 2 μg of histamine per 10^6 cells. The total histamine content was 20 ± 3 μg per 10^6 cells. The release of 3-methylhistamine in supernatants from stimulated peritoneal mast cells was not observed. Analysis of supernatants from stimulated peritoneal mast cells, rat heart homogenates and coronary perfusates on days 1 and 7 resulted in amounts identical with those observed on day 0.

DISCUSSION

Although many HPLC methods to determine histamine in biological samples have been described [7–14], none of these combines purification and concentration of samples followed by derivatization with fluorescamine. OPA, most commonly used as the derivatizing agent for histamine [20], requires carefully controlled reaction conditions and duration of the derivatization reaction. Moreover, the derivatization products have a limited stability ($t_{1/2}$ being *ca.* 3 h, calculated from ref. 6). Post-column derivatization requires expensive additional equipment for the chromatographic system. In addition, the use of a post-column reagent results in peak broadening and dilution of the samples [6]. Therefore, Saito *et al.* [6] proposed the use of an on-column derivatization method with the mobile phase containing the OPA reagents. However, this on-column derivatization method requires an expensive HPLC set-up to optimize the chromatographic conditions.

The present method describes the determination of histamine and 3-methylhistamine in biological samples using fluorescamine as the derivatizing agent. Fluorescamine reacts with compounds possessing a primary amino group almost instantaneously at room temperature in

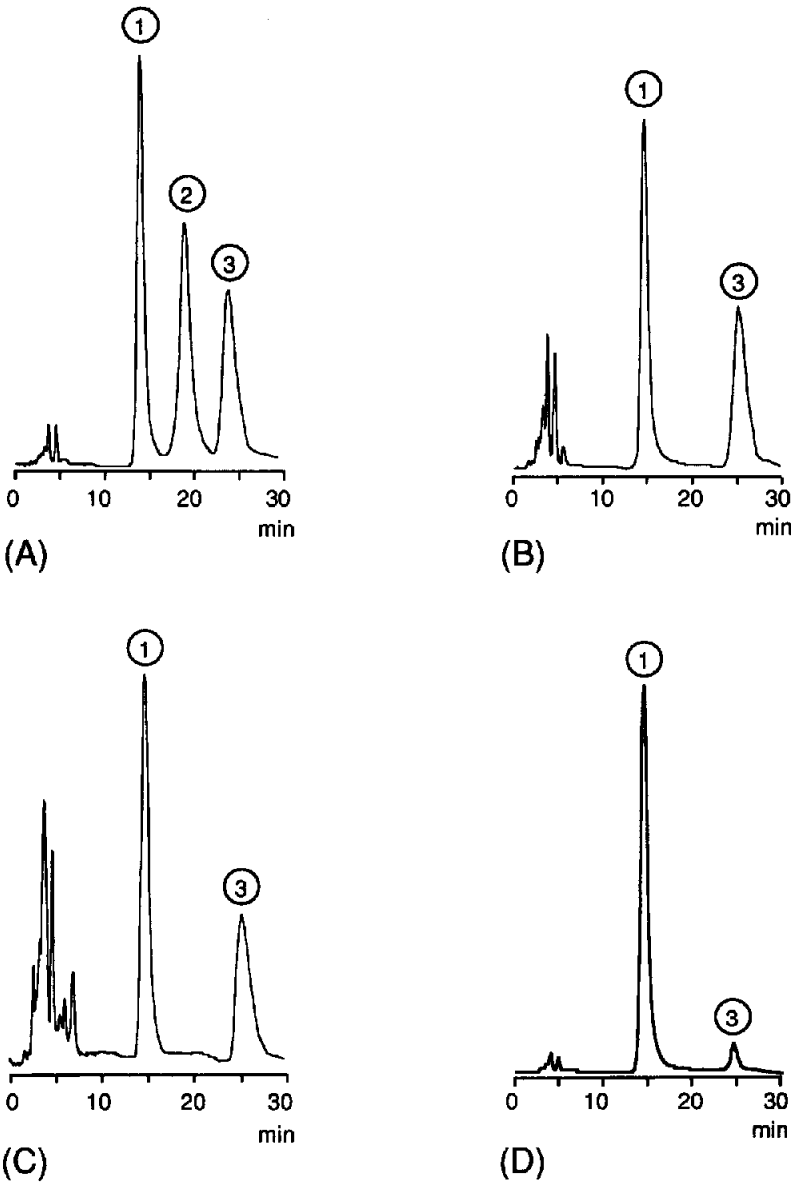

Fig. 1. Typical reversed-phase chromatograms of standards and biological samples: (A) standard fluorescamine derivatives, each peak representing 5 ng on column; (B) coronary perfusate from a heart stimulated with 50 μg of the mast cell degranulator compound 48/80; (C) an extract of a rat heart homogenate; (D) supernatant of rat peritoneal mast cells stimulated with 10 μg/ml compound 48/80 in the presence of 1 m*M* $CaCl_2$. Peaks: 1 = histamine; 2 = 3-methylhistamine; 3 = internal standard. The *y*-axis represents fluorescence intensity (arbitrary units), the *x*-axis represents the retention time (min). Conditions: column, Chrompack RP18 (250 × 4 mm I.D.); mobile phase, acetonitrile–water–triethylamine–H_3PO_4 (850:150:4:4, v/v), pH 6.4 with NH_4OH; flow-rate, 0.7 ml/min; column temperature, 20°C; excitation wavelength, 360 nm; emission wavelength, 440 nm.

aqueous solution at pH 7.5–9 [21]. The excess of fluorescamine is subsequently hydrolysed ($t_{1/2}$ *ca.* 10 s) to yield a water-soluble non-fluorescent product. Fluorescamine has the advantage that all samples can be derivatized simultaneously prior to HPLC injection. Moreover, there is no need to control the derivatization time. We demonstrated that fluorescamine-derivatized samples

were relatively stable over a period of 7 days. Reanalysis of samples on day 7 demonstrated that absolute peak areas of histamine, 3-methylhistamine and 1-methylhistamine on day 7 were *ca.* 90% of those observed on day 0. The inclusion of the internal standard corrects for the relatively small decrease in fluorescence. Because the relative peak areas of histamine and 3-methylhistamine on days 1 and 7 (samples used for the calibration curves) were not significantly different from those observed on day 0 for all the amounts investigated, it can be concluded that samples can be analysed up to 7 days after derivatization without significant loss of accuracy. The method described has a good reproducibility (Table I). Because the absolute peak areas of samples concentrated on cation-exchange columns were always more than 95% of the unconcentrated samples, the procedure allows a clean-up/concentration procedure without a substantial loss of material. Very small amounts of histamine and 3-methylhistamine can be determined because the method has a low detection limit (20 pg on column, signal-to-noise ratio 3:1).

The described method was used to assess the histamine and 3-methylhistamine content of various biological samples. We demonstrated that *ca.* 50% of the histamine present in rat hearts is released during the first minute after stimulation of the cardiac mast cells with compound 48/80. This has also been reported by Wan *et al.* [22]. 3-Methylhistamine was undetectable in coronary perfusates from rat hearts stimulated with compound 48/80. Total histamine in rat hearts was found to be 3.7 ± 0.4 μg/g wet weight (mean ± S.D.), a value that has been found earlier [17]. Interestingly, we were unable to demonstrate the presence of 3-methylhistamine in rat hearts, which is in contrast to the previous results. Kasziba *et al.* [17] demonstrated the presence of histamine and 3-methylhistamine in amounts of 4.5 ± 0.2 and 14.5 ± 1.1 μg/g wet weight, respectively. The reason for this discrepancy is unclear. When samples of rat heart homogenates were spiked with 3-methylhistamine, we observed an average recovery of 96% of this compound. This demonstrates that 3-methylhistamine can be determined accurately by the described method. Total histamine of rat peritoneal mast cells was found to be 20 ± 3 μg per 10^6 cells (n = 3); 90% of the histamine present could be released by stimulation with 10 μg/ml compound 48/80. These values have been reported earlier [23,24]. The release of 3-methylhistamine by stimulated peritoneal mast cells was not observed.

CONCLUSION

A sensitive and rapid isocratic HPLC assay for the determination of histamine and 3-methylhistamine in biological samples using fluorescamine as the derivatizing agent is described. The method has the advantage that all samples can be derivatized simultaneously and analysis can be carried out in a sample, automated low-cost HPLC system. Because the derivatives are relatively stable over a period of 7 days, samples need not necessarily be analysed immediately after derivatization.

ACKNOWLEDGEMENTS

The authors thank J. Wierts, from the Department of Human Biology, University of Limburg, for his excellent technical assistance in obtaining the peritoneal mast cells and the care of the animals. Dr. J. Heemskerk from the Department of Biochemistry, University of Limburg, is acknowledged for his assistance in the experiment with the peritoneal mast cells. Dr. T. Ambergen from the Department of Medical Informatics and Statistics, University of Limburg, is acknowledged for his advice in the statistical analysis of the data.

REFERENCES

1 G. Marone, G. Ambrosio, D. Bonaduce, A. Genovese, M. Triggiani and M. Condorelli, *Int. Arch. Allergy Appl. Immunol.*, 74 (1984) 356.
2 E. S. Schulman, D. W. MacGlashan Jr., S. P. Peters, R. P. Schleimer, H. H. Newball and L. M. Lichtenstein, *J. Immunol.*, 129 (1982) 2662.
3 P. A. Shore, A. Burkhalter and J. V. H. Cohn, *J. Pharmacol. Exp. Ther.*, 127 (1959) 182.

4 J. R. Vane, *Br. J. Pharmacol.*, 20 (1964) 360.
5 H. Mita, H. Yasueda and T. Shida, *J. Chromatogr.*, 221 (1980) 1.
6 K. Saito, M. Horie, N. Nose, K. Nakagomi and H. Nakazawa, *J. Chromatogr.*, 595 (1992) 163.
7 Y. Arakawa and S. Tachibana, *Anal. Biochem.*, 158 (1986) 20.
8 A. Bettero, M. R. Angi, F. Moro and C. A. Benassi, *J. Chromatogr.*, 310 (1984) 390.
9 S. P. Ashmore, A. H. Thomson and H. Simpson, *J. Chromatogr.*, 496 (1989) 435.
10 G. Skofitsch, A. Saria, P. Holzer and F. Lembeck, *J. Chromatogr.*, 226 (1981) 53.
11 A. L. Rönnberg, C. Hansson and R. Håkanson, *Anal. Biochem.*, 139 (1984) 338.
12 J. Y. Hui and S. L. Taylor, *J. Chromatogr.*, 312 (1984) 443.
13 J. C. Robert, J. Vatier, B. K. N. Phuoc and S. Bonfils, *J. Chromatogr.*, 273 (1983) 275.
14 A. Yamatodani, H. Fukada, H. Wada, T. Iwaeda and T. Watanabe, *J. Chromatogr.*, 344 (1985) 115.
15 E. Kownatzki, G. Gruninger and N. Fuhr, *Pharmacology*, 34 (1987) 17.
16 W. Lorenz, K. Than, E. Neugebauer, H. Stoltzing, C. Ohmann, D. Weber, A. Schmal, E. Hinterlang, H. Barth and J. Kusche, *Agents Actions*, 21 (1985) 1.
17 E. Kasziba, L. Flancbaum, J. C. Fitzpatrick, J. Schneiderman and H. Fischer, *J. Chromatogr.*, 432 (1988) 315.
18 L. Flancbaum, J. C. Fitzpatrick and H. Fisher, *Circ. Shock*, 27 (1989) 155.
19 C. M. C. J. van Haaster, W. Engels, P. J. M. R. Lemmens, G. Hornstra and G. J. van der Vusse, *Biochim. Biophys. Acta*, 1167 (1993) 147.
20 R. Håkanson, A. L. Rönnberg and K. Sjölund, *Anal. Biochem.*, 47 (1972) 356.
21 C. Y. Lai, *Methods Enzymol.*, 47 (1977) 236.
22 B. Y. C. Wan, K. H. Peh and E. S. K. Assem, *Agents Actions*, 33 (1991) 71.
23 S. J. Galli, A. M. Dvorak and H. F. Dvorak, *Progress Allergy*, 34 (1984) 90.
24 J. Dainaka, A. Ichikawa, M. Okada and K. Tomita, *Biochem. Pharmacol.*, 33 (1984) 1653.