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Short communication

Aspirin inhibits collagen-induced platelet serotonin release, as measured by microbore high-performance liquid chromatography with electrochemical detection

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Abstract

A sensitive microbore high-performance liquid chromatographic method with electrochemical detection has been developed for the measurement of small quantities of serotonin (5-hydroxytryptamine; 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in rabbit platelets. The limit of detection of 5-HT is 0.1 ng/ml. To evaluate the 5-HT release from platelet suspension, the aggregating agent collagen was added at concentrations of 10, 30 and 100 μg/ml and the 5-HT concentrations rose from the base level (11.80 ± 0.42 ng/ml) to 123.97 ± 11.02, 361.96 ± 17.90 and 470.45 ± 35.46 ng/ml, respectively. Further results demonstrated that aspirin inhibits collagen-induced 5-HT release from platelet suspension significantly.

1. Introduction

Aspirin (acetylsalicylic acid) inhibits platelet aggregation. The labile acetyl residue binds covalently to cyclooxygenase in platelets, resulting in irreversible inhibition of aggregation and prevention of thromboxane A2 synthesis [1]. For the 5-HT determination, spectrofluorometry is commonly used; however, its detection limit is low [2]. Radioimmunoassay and enzymatic assay can increase sensitivity [3,4]. A new procedure which consists of labelling the granule-bound pool by incorporation of radioactive 5-HT, then measuring the appearance of radioactivity extracellularly to quantify 5-HT secretion, has been developed [5]. However, all of these methods are complicated and time-consuming. Recently, various ways of determining 5-HT in blood, platelets [6], platelet-rich plasma [7–10], platelet-deficient or platelet-free plasma [11,12] have been reported. The aim of this study was to develop a simple, sensitive procedure based on microbore high-performance liquid chromatography (HPLC) with electrochemical detection

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(ED) for the measurement of 5-HT in rabbit platelets. In addition, the anti-platelet effect of aspirin in collagen-induced 5-HT release was determined.

2. Experimental

2.1. Materials

Aspirin, 5-HT, 5-HIAA and collagen (type I, bovine achilles tendon) were purchased from Sigma (St. Louis, MO, USA). HPLC reagents and buffer reagents were obtained from Merck (Darmstadt, Germany). Triple-deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Platelet suspension

Rabbit blood collected from the marginal ear vein was mixed with one sixth by volume of acid citrate dextrose. The blood was centrifuged by swinging centrifugation at 200 *g* for 15 min at room temperature. The upper platelet-rich plasma was mixed with 2 m*M* EDTA and centrifuged at 1000 *g* for 12 min. The supernatant was discarded and the platelet pellet was suspended in Ca^{2+}-free Tyrode's buffer (136.89 m*M* NaCl, 2.68 m*M* KCl, 2 m*M* $MgCl_2$, 0.33 m*M* NaH_2PO_4, 5 m*M* glucose, 10 m*M* HEPES [4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid)]) with 0.35% bovine serum albumin, heparin (50 unit/ml) and apyrase (1 unit/ml). After 20 min incubation at 37°C, the washed platelet pellet was resuspended in Tyrode's buffer containing 1 m*M* calcium and the cell concentration was adjusted to around $1 \cdot 10^8$ platelets/ml. The drug-treatment reaction was terminated after 3 min by mixing the sample with one-fifth by volume of 0.05 m*M* EDTA in ice. After centrifugation at 10 000 *g* for 3 min, the supernatant was filtered through a 0.2-μm membrane filter. A 10-μl aliquot of the filtrate was injected directly onto the HPLC apparatus for analysis.

2.3. Apparatus and chromatography

The HPLC–ED system consisted of a syringe pump (ISCO, Lincoln, NE, USA) with the flow-rate set at 0.06 ml/min for 5-HT analysis (Fig. 2). Samples were separated using a reversed-phase C-18 SepStik microbore column (150 × 1 mm, 5 μm; BAS, West Lafayette, IN, USA) fitted with a microbore guard column (14 × 1 mm, 5 μm; BAS). The injection volume was configured with a 10-μl sample loop. The mobile phase consisted of 110 ml acetonitrile, 2.08 m*M* sodium 1-octanesulfonate, 13.48 m*M* monosodium dihydrogen orthophosphate, 56.59 m*M* sodium citrate, 0.027 m*M* EDTA, and 1 ml diethylamine. The final volume of the mobile phase was added to 1 l of triple-deionized water. The solution was adjusted to pH 3.0 by orthophosphoric acid. The mixture was filtered with a 0.22-μm Millipore membrane. 5-HT and 5-HIAA were measured using an amperometric detector (BAS-4C) coupled to a glassy carbon working electrode and referenced to a Ag/AgCl electrode at +0.6 V. The output from the ED was amplified and recorded using Waters Millennium 2010 software [13].

2.4. Statistical analysis

All results are expressed as the mean ± S.E.M from six experiments. Statistical analysis was performed by the Student–Newman–Keuls test with the level of significance set at $p < 0.01$.

3. Results and discussion

The method described in this paper gives excellent separation of 5-HT and 5-HIAA. The system has a number of major advantages over those previously reported [2–5]. First, the microbore column increases the sensitivity to a compound, decreasing band broadening so that sharper peaks are obtained. Furthermore, the analysis time is shortened from approximately 20 min to < 10 min [9,10,13]. Moreover, only small quantities of sample are required. In addition,

Table 1
Intra- and inter-assay precision and accuracy in 5-HT determination

	Nominal concentration (ng/ml)		
	5	20	100
Intra-assay			
Mean ($n = 5$)	5.23	19.47	100.26
S.D.	0.31	0.30	0.31
% C.V.	5.85	3.45	0.31
% Accuracy	4.60	−2.65	0.26
Inter-assay			
Mean ($n = 5$)	5.18	19.29	101.57
S.D.	0.59	0.48	3.82
% C.V.	11.4	2.51	3.76
% Accuracy	3.64	−3.56	0.57

Precision (% C.V.) = (standard deviation/mean concentration) · 100; accuracy (%) = [(mean conc. − actual conc.)/actual conc.] · 100.

the microbore HPLC system is easy to maintain and can be used to provide rapid measurements of platelet 5-HT.

The calibration curve was linear for 5-HT (range 1 to 400 ng/ml). The linear regression of 5-HT is $y = 2.13 \cdot 10^{-6}x + 0.28$ ($r = 1$), where y is the concentration in ng/ml and x is the response in peak area. Intra- and inter-assay precision and accuracy were determined at three different concentrations. The results are presented in Table 1. Peak detection limits for 5-HT and 5-HIAA were about 0.1 ng/ml at a signal-to-noise ratio of 4.

Fig. 1A shows typical chromatograms of a standard mixture containing 10 ng/ml of 5-HT and 5-HIAA. The retention times were 5.5 and 7.0 min, respectively. Fig. 1B shows the base 5-HT of untreated platelet suspension. No peaks were discernible within the time window in which 5-HT (10.71 ng/ml) and 5-HIAA (0.82 ng/ml) were detected. Fig. 1C shows the collagen-induced 5-HT release: 114.37 ng/ml at a collagen concentration of 10 μg/ml. Fig. 1D shows that, at the same collagen concentration, the 5-HT release was reduced to 51.77 ng/ml in the presence of 100 μM aspirin. The base platelet 5-HT concentration was 11.80 ± 0.42 ng/ml. The concentrations following 5-HT release were 123.97 ± 11.02, 361.96 ± 17.90 and 470.45 ± 35.46 ng/ml, induced by 10, 30 and 100 μg/ml collagen, respectively.

Fig. 2 shows that collagen (10, 30 or 100 μg/ml) induced 5-HT release from platelet suspension and that aspirin (100 μM) inhibited collagen-induced 5-HT release.

Serotonin is a rather weak direct activator of platelets, causing only a shape change and a

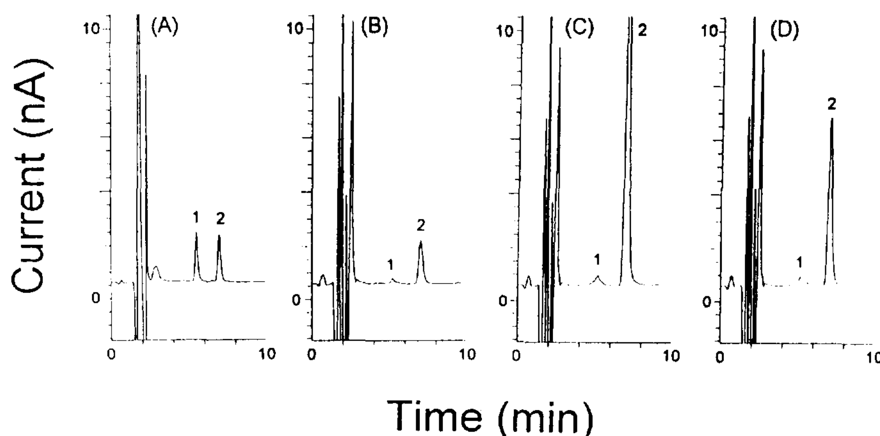

Fig. 1. Typical chromatograms of (A) a standard mixture of 5-HT (10 ng/ml) and 5-HIAA (10 ng/ml), (B) a baseline of platelet 5-HT (10.71 ng/ml) and 5-HIAA (0.82 ng/ml), (C) collagen-induced platelet 5-HT (114.37 ng/ml) release, and (D) aspirin-inhibited collagen-induced 5-HT (51.77 ng/ml) release. 1 = 5-HIAA; 2 = 5-HT.

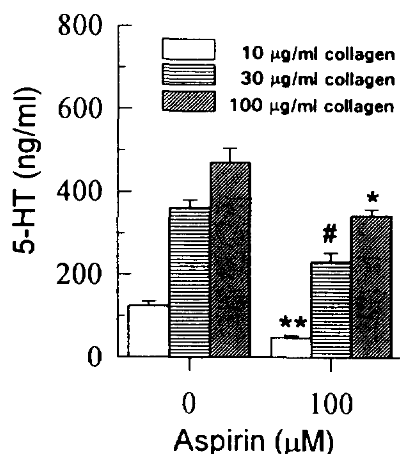

Fig. 2. Collagen-induced 5-HT release from platelet suspension ($n = 6$): $p < 0.01$ vs. collagen.

modest, transient aggregation reaction of little functional significance. However, it potently augments the platelet responses to other activators such as ADP, collagen, or thromboxane A_2. The serotonin-induced amplification of platelet aggregation can be prevented by $5\text{-}HT_2$ serotonergic blockers (e.g. ketanserin) [14–17].

The major activation of platelets induced by collagen is due to the formation of thromboxane A_2 [18,19]. Moreover, the thromboxane A_2-triggered secretion is mediated by increased intracellular calcium [20]. In addition, intracellular calcium increase has been regarded as the final common pathway for platelet shape change, secretion and aggregation [21].

Serotonin displays complex properties in relation to vascular tissues, producing constriction in venules and dilation in arterioles. However, the relationships between serotonin content and platelet function in the initiation and maintenance of vascular function are poorly understood [14]. The rapid measurement of platelet 5-HT by the microbore HPLC–ED system may prove to be a useful tool in this area of investigation.

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